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Reprogramming the Immune System with Anti-CD4 Monoclonal Antibodies

Joana Duarte Nunes Duarte

Tese orientada por Professor Doutor Luís Graça
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Em memória do meu querido avô Duarte.
Com muita saudade.

“What you have given, you can no longer lose.”

Leonard Peltier

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This is it!!!

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Abbreviation List

2D2 – MOG-specific TCR-transgenic mice

AHR – airway hiperresponsiveness

AICD – activation-induced cell death

AIRE – autoimmune regulator gene

APC - Antigen Presenting Cell

BBB – blood brain barrier

BCR – B cell receptor

CDR – complementarity determining region

CFA – complete Freund's adjuvant

CIA – collagen-induced arhtitis

CII – collagen II

CLN – cervical lymph nodes

CNS – central nervous system

CPE – crude peanut extract

CpG – cytosine guanine dinucleotide

CTLA-4 – cytotoxic T cell associated antigen

DAMP – danger-associated molecular pattern

DC - Dendritic Cell

DMARD – disease-modifying anti-rheumatic drug

DN – double negative

DTH – delayed type hypersensitivity

EAE – experimental autoimmune encephalomyelitis

GA – glatiramer acetate

GITR – glucocorticoid-induced TNF related protein

G6PI – glucose-6-phosphoisomerase

GM-CSF – granulocyte-macrophage colony-stimulating factor

HLA – human çeucocyte antigen

IDO – indoleamine 2,3-dyoxigenase

iTreg – induced Treg

Ig – immunoglobulin

IFA – incomplete Freund's adjuvant

IFN – Interferon

IL – interleukin

i.n. – intranasal

i.p. – intraperitoneal

i.v. - intravenous

K/BxN – G6PI transgenic TCR mice

KO – knock out

LAG – Lymphocyte activation gene

LFA - Lymphocyte Function-Associated Antigen

LN - Lymph Nodes

LPS - Lipopolysaccharide

Mab – monoclonal antibody

MBP – myelin-basic protein

MCP-1 – monocyte chemotactic protein-1

MHC - Major Histocompatibility Complex

MOG – myelin oligodendrocyte glycoprotein

MS – multiple sclerosis

MTP – metatarso-phalangical

MTX - methotrexate

MuHV-4 – muride herpesvirus-4

NO – nitric oxide

NOD – non-obese diabetic

NK – natural killer

NKT – natural killer T cells

nTreg – natural Tregs

ODC – oligodendrocytic cell

OVA - ovalbumin

PAMP – pathogen associated molecular patterns

PMA -phorbol 12-myristate 13-acetate

Pfu – plaque forming units

PLP – proteolipid protein

PRR – pattern recognition receptor

RA - rheumatoid arthritis

RANKL - Receptor Activator for NFκB Ligand

RF – rheumatoid factor

ROS – reactive oxygen species

s.c. – subcutaneous

SCID - Severe Combined Immunodeficiency

SPF – specific pathogen free

T1D – type-1 diabetes

TCR – T cell receptor

TEC – thymic epithelial cell

TLR – Toll-like receptor

TGF - Transforming Growth Factor

Th – Thelper cells

TNF - Tumor Necrosis Factor

Tr1 – IL-10 producing regulatory T cells

TR- - RAG deficient MBP-specific TCR-transgenic mice

Tregs – regulatory T cells

VLA-4 – very late antigen-4/α4β1 integrin

WT – wild type

Sumário

A tolerância imunitária é o estado em que o sistema imunitário não responde agressivamente contra um determinado conjunto de antigénios, permanecendo competente para proteger o organismo de invasores patogénicos. Geralmente, o sistema imunitário é tolerante aos nossos próprios antigénios (auto-antigénios), a antigénios alimentares (tolerância oral) e a muitas outras substâncias externas às quais estamos regularmente expostos (por exemplo pollens e outros potenciais alergénios). No entanto, sob determinadas condições (ambientais ou genéticas) pode haver uma quebra desse estado de tolerância a determinados antigénios, originando patologias como doenças autoimunes ou alérgicas. As células T reguladoras (Tregs) são elementos fulcrais na manutenção da tolerância periférica, desempenhando um papel crucial na prevenção de reacções autoimunes, bem como respostas de hipersensibilidade.

Os mecanismos moleculares que caracterizam a supressão mediada por Tregs são ainda pouco claros, e a investigação dos mesmos é uma prioridade, pois pode revelar alvos importantes para intervenção terapêutica. Estudos em ratinhos revelaram que a utilização de anticorpos monoclonais, dirigidos para moléculas chave expressas em linfócitos, levaram à indução de tolerância a longo prazo, após um período terapêutico reduzido. Este conceito ficou conhecido como reprogramação do sistema imunitário ou indução de tolerância terapêutica. Estudos em transplantação demonstraram que o anticorpo não depletante anti-CD4 leva a tolerância a longo prazo, através da indução de Tregs. Aqui descrevo a minha investigação sobre o impacto de anti-CD4 como terapêutica em diferentes doenças imuno-mediadas, com o objectivo de reprogramar o sistema imunitário induzindo tolerância.

Primeiro, estudei a indução de tolerância em modelos de animais de doenças autoimunes, onde a tolerância a auto-antigénios foi de alguma forma afectada. Aproveitámos a existência de modelos animais bem estabelecidos, tanto de artrite autoimmune como de esclerose múltipla, que se sabem ser mediados por células Th1 e Th17, e portanto fazem do CD4 um bom alvo terapêutico. O tratamento com anti-CD4 foi bem sucedido em ambos os modelos, prevenindo o desenvolvimento de ambas as doenças, e impedindo a progressão das mesmas quando estabelecidas. Investigámos o mecanismo que caracterizava a protecção por anti-CD4, e percebemos que este baseia-se maioritariamente em re-estabelecer o equilíbrio entre as células efectoras e as células reguladoras, favorecendo a tolerância. Em artrite autoimune este efeito verificou-se a nível local, na zona de inflamação (sinóvia), onde as células Th17 estavam claramente reduzidas, enquanto as Tregs eram significativamente superiores àquelas dos animais

tratados. O estudo de encefalomielite autoimune experimental (EAE) permitiu-nos localizar as células T específicas para o antigénio, e consequentemente estudar o impacto do anti-CD4 em células naïve e pré-activadas. O anti-CD4 inibiu a proliferação e diferenciação das células T naïve para células efectoras produtoras de citocinas pro-inflamatórias (como IL-17 e IFN-γ), com uma acumulação progressiva de células T reguladoras a longo prazo, que demonstrámos ser importantes na manutenção da tolerância adquirida. Além disso, a terapia com anti-CD4 afecta as células pré-activadas de forma diferente, induzindo a apoptose das mesmas, mais uma vez favorecendo o raio de células efectoras/células reguladoras no sentido da tolerância. De relevância, a tolerância induzida é específica para o antigénio presente na fase do tratamento, permitindo aos animais tolerantes permanecerem competentes para desenvolver uma resposta protectora contra um antigénio diferente, como no caso da resolução de uma infecção viral.

Uma vez estabelecida a eficácia da tolerância induzida pelo anti-CD4 em doenças mediadas por células Th1 e Th17, avaliei o impacto desta mesma terapia num modelo de doença grave mediado por células Th2. Para isto, utilizámos um modelo animal bem estabelecido de choque anafilático induzido por amendoins, em ratinhos C3H/HeJ. O tratamento com anti-CD4 durante a fase de exposição a extracto de amendoim induziu a protecção perante nova sensibilização a amendoim, inibindo o desenvolvimento de manifestações de doença anafilática. Esta protecção revelou-se mais uma vez específica para o antigénio em questão, permitindo que o sistema imunitário respondesse normalmente na presença de um antigénio diferente, inclusivamente, através da produção de IgE e citocinas do tipo Th2. A tolerância induzida pareceu ser dependente de Tregs, uma vez que os animais tratados com anti-CD4 exibiram níveis significativamente elevados de Foxp3, e aquando da deplecção de células CD25+ durante o tratamento com anti-CD4, não foi possível induzir o estado de tolerância.

Em conclusão, os resultados obtidos nesta tese sugerem que anticorpos monoclonais anti-CD4 têm um potencial terapêutico abrangente, para tratamento de patologias mediadas pelos diferentes subtipos de células T CD4, sejam doenças autoimunes ou alérgicas.

Summary

Immune tolerance is a state where the immune system does not respond aggressively towards a set of antigens while remaining fully competent to mount protective responses. The immune system is usually tolerant to our own antigens (self), to food antigens (oral tolerance), and to several other foreign substances to which we are regularly exposed (such as pollens and other potential allergens). Nevertheless, under certain conditions (genetic or environmental) there is a breakdown of tolerance to certain antigens, thus originating the onset of autoimmune and allergic pathologies. Regulatory T cells (Tregs) are central players in the maintenance of peripheral tolerance, having an essential role in preventing autoimmunity, as well as hypersensitivity reactions. However, the molecular mechanisms which mediate suppression are still obscure, and their investigation is a current priority, as it may reveal important targets for immune intervention. Studies in mouse models show that monoclonal antibodies (mAbs) targeting key lymphocyte molecules are able to produce long-term tolerance following a short-term therapy. This concept became known as immune reprogramming or therapeutic tolerance induction. Non-depleting anti-CD4 mAb have been shown to induce long term tolerance in transplantation through induction of Treg cells. Here I describe my research on the impact of non-depleting anti-CD4 mAb in different immune-mediated pathologies aiming to reprogram the immune system towards tolerance induction. Furthermore, I studied the cellular and molecular mechanisms that mediate tolerance induction.

The first step in this study was to assess tolerance induction in murine models of autoimmune diseases, where self-tolerance is broken. We took advantage of well established animal models of rheumatoid arthritis (RA) and multiple sclerosis (MS), which are known to be mediated by Th1 and Th17 cells, thus indicating CD4 would be an optimal therapeutic target. Treatment with anti-CD4 was successful in both models, being able to prevent the onset of the disease, and impairing disease progression. We found the mechanism characterizing anti-CD4 effect relies on resetting the balance between effector and Treg cells towards a tolerance-favoring ratio. In autoimmune arthritis we found this effect to be especially evident locally at the site of inflammation (within the synovia), where Th17 effector cells are markedly reduced and the Treg frequency is increased in anti-CD4 treated mice. The study of experimental autoimmune encephalomyelitis (EAE) allowed us to track antigen-specific T cells, and further study the impact of anti-CD4 treatment on naïve and pre-activated T cells. We found anti-CD4 prevented the proliferation and differentiation of naïve T cells into effector cells producing pro-inflammatory cytokines (such as IL-17 and IFN-γ), with a progressive

accumulation of Treg cells at a later time that are important in maintaining long-term protection from the disease. Furthermore, anti-CD4 therapy targets pre-activated T cells in a different way by committing effector T cells towards apoptosis, thus leading to a ratio between effector and regulatory cells that once again favors tolerance. Importantly, the reprogramming is specific for the antigens present at the time of anti-CD4-treatment, with tolerant mice remaining fully competent to mount protective immune responses, namely to eradicate viral infections.

Having established the efficacy of anti-CD4-induced tolerance induction in Th1/Th17 mediated diseases, I wanted to evaluate the impact of anti-CD4-treatment in a stringent model of Th2-induced pathology, studying whether the mAb treatment could prevent peanut-induced anaphylaxis in C3H/HeJ mice. Treatment with anti-CD4 at the time of exposure to peanut antigens led to long-term protection from further sensitization with peanut-antigens and the development of anaphylaxis. Such long-term tolerance was antigen specific as mice remained competent to respond to different antigens, namely by producing Th2-mediated responses leading to IgE production. We found that long-term tolerance appeared to be dependent on Treg cells: not only anti-CD4 treated mice exhibited an increased frequency of Foxp3+ Tregs, but also CD25 depletion at the time of anti-CD4 treatment abrogated tolerance induction.

Taken together the results presented in this thesis suggest CD4 is a promising therapeutic target for the treatment of immune-mediated pathologies, as different as autoimmune and allergic diseases.

General Introduction

1. General Introduction

1.1. The Immune System

Throughout the years, the immune system has evolved to protect the host from a broad range of pathogenic microbes, which also evolve constantly. To perform these functions properly, it is essential to be able to distinguish self from non-self.

Both innate and adaptive immunity are important to eliminate invading pathogens. The Innate immune system is composed of a large number of different cell subsets, with broad expression of distinct recognition molecules (pattern recognition receptors – PRRs), leading to a fast and unspecific activity against any pathogen, constituting the first line of host response. The Adaptive immune response is specific for each individual pathogen, and starts with lower number of cells, which will activate and proliferate upon encountering the antigen, and mount an efficient immune response against it. So, generally, a primary adaptive immune response starts later than innate immune response in host defense. One of the main features of adaptive immunity, besides its specificity, is that once the cells encounter an antigen for the first time, they will generate memory cells which persist in the organism in a steady state, and will later allow the host to respond more rapidly when exposed to the same antigen, even if this re-encounter happens decades later (secondary response). This memory response is the basis for successful vaccination.

1.1.1. Innate Immune Response

The innate immune response is crucial for an effective host defense. This type of response, even though lacking antigen-specificity, has the ability to discriminate between dangerous pathogens and innocuous or even beneficial microbes and environmental factors, due to PRRs. Speed is a defining characteristic of the innate immune system, within minutes of pathogen exposure, it starts generating a protective inflammatory response. Moreover innate immunity plays a central role in activating the subsequent adaptive immune response.

Innate immunity augments the protection offered by anatomic and physiological barriers[1]. Part of the constitutive mechanisms in an innate immune system are the physical barriers like cell-cell tight junctions or secreted mucus layer in the respiratory, gastrointestinal and genitourinary tracts. But most importantly the innate immune system is armed with potent cells from both hematopoietic and nonhematopoietic origin.

Hematopoietic cells involved in innate response include macrophages, dendritic cells (DCs), mast cells, neutrophils, eosinophils, natural killer (NK), and NKT cells. Some of these cells act by engulfing pathogenic microbes into intracellular vacuoles, where they are exposed to toxic molecules, such as NO, superoxide, and degradative enzymes that will destroy the microorganism. Neutrophils are able to release large quantities of reactive oxygen species (ROS) highly cytotoxic for bacterial pathogens, and produce enzymes which are important in tissue remodeling[2]. Macrophages are responsible for the release of highly proinflammatory cytokines, such as IFN-γ, IL-6, IL-12 and TNF, holding important antibacterial activities[3]. Eosinophils, basophils and mast cells, are usually associated to immune responses against parasites, like in the case of Helminths infections, as well as in allergic responses[4,5]. Mast cells and basophils act through the release of histamine and other lipid mediators that will stimulate tissue inflammation and smooth muscle contraction. NK cells destroy target cells through the release of apoptosis-inducing molecules like granzymes and perforin, or by antibody-dependent cytotoxicity. They have prominent antitumor effects and are potent killers of viral infected cells[6]. Besides cellular responses innate immunity also has a humoral component, including well characterized components like complement proteins, LPS-binding protein, C-reactive protein and other pentraxins, collectins and antimicrobial peptides, including defensins[7]. These circulating proteins are involved in sensing microbes and effector mechanisms to facilitate clearance of the infection.

There are three main strategies that underlie innate immunity response way of action. First, innate immunity relies on a limited repertoire of germline-encoded receptors (pattern recognition receptors – PRRs) to recognize “microbial non-self”, conserved molecular structures that are expressed by a large variety of microbes (pathogen associated molecular patterns-PAMPs). This was first postulated in 1989 by Janeway, who said that a class of pattern recognition molecules must exist that function as an initial defense against infection, by rapidly detecting conserved molecular features by pathogens[8]. Several classes of PRRs have been described, namely families of Toll-like receptors (TLRs)[9], which are now extended to 10 different receptors[10], and are usually expressed in antigen presenting cells like macrophages and DCs[11]. The TLR family detects a broad range of PAMPs, for instance, TLR-4 can be activated by LPS, while TLR-2 recognizes zymosan (an yeast cell wall component), TLR-3 is activated by ds-RNAs from viruses, and TLR-9 recognizes cytosine-guanin dinucleotide (CpG) DNA motifs[12]. Another strategie used by the innate immune response, was first postulated by Matzinger[13] in 1994, who proposed that cellular damage is a critical factor underlying immune activation. Throughout the years several danger-associated molecular patterns (DAMPs)

have been identified, which result in the activation of host stress response pathways, including products of necrotic cells, perturbation of intracellular ion gradients, and generation of ROS[14]. The third mechanism used by the innate immune response, are the receptors that recognize molecules expressed by normal healthy cells, and not microbes or infected cells, delivering an inhibitory signal to prevent activation against host tissues[15].

Upon stimulation PRRs and danger receptors activate signaling pathways that constitute the front lines of host defense against pathogen infection, and can lead to cell autonomous immune responses like autophagy, to the induction of programs that restrict viral replication, to the production of proinflammatory cytokines and chemokines. Ultimately these signals will help direct the adaptive immune response[16].

It is well known that activation of TLRs on antigen presenting cells (APCs) initiates a cascade of intracellular events, which will enhance antigen presentation, production and release of pro-inflammatory cytokines and upregulation of adhesion and co-stimulatory molecules which are all central for adaptive immune system priming[17,18]. Different types of DCs, selectively express cytokines, co-receptors and several other polarizing signals that promote the development of Th1, Th2, Th17 or even Tregs[19,20]. TLRs importance in adaptive immune system is becoming increasingly evident as it has been shown that T cells can also express certain types of TLRs[21], and they can also function as co-stimulatory receptors enhancing effector T cell proliferation, survival, and cytokine production[22]. Thus, it is becoming clear that innate immunity cannot be considered exclusively as the "nonspecific immunity", as it is an important partner of adaptive immunity.

1.1.2. Adaptive Immune Response

Even though the innate immune system provides critical mechanisms for the rapid sensing and elimination of pathogens, the adaptive immune system has evolved to provide a broader and more finely tuned repertoire of recognition of both self and non-self antigens. The adaptive immune response is characterized by its enormous diversity in antigen recognition, high antigen specificity, potent effector activity, and long-lasting immunologic memory. It is mainly mediated by T and B lymphocytes, that develop in the primary lymphoid organs (thymus and bone marrow, respectively), and then traffic to the secondary lymphoid organs (namely, lymph nodes and spleen), which capture circulating antigens from lymph and blood. T and B cells are characterized by the expression of T-cell receptor (TCR) and B-cell receptor (BCR), respectively, which are responsible for the specificity conferred to this type of immune response. These receptors are encoded by

genes that are assembled by somatic rearrangements of germline gene elements, permitting the generation of millions of different receptors, each one with an unique ability to recognize a specific antigen.

Although the innate and adaptive immune systems are often described as independent arms of the immune system, they usually act together. Thus, components from the innate immune system contribute to the activation of T and B cells, and on their turn, antigen-specific lymphocytes amplify the response by recruiting innate immune cells as effector components to help controlling pathogenic invasion.

APCs of the monocyte/macrophage lineage are one of the major examples of the importance of innate immune cells for adaptive immune responses. These cells take up the pathogen antigens and process them, through proteolysis, into peptide fragments, presenting them within a major histocompatibility complex (MHC) molecule (encoded in the human leucocyte antigen (HLA) loci), and activating T cell responses. Although all nucleated cells can present antigens in the context of MHC class I, only APCs express MHC class II, a group that includes B cells. DCs are considered the most potent APCs, being distributed all over the body, and concentrated in secondary lymphoid tissues. Specialized cells from this lineage can be found in different locations, like Langerhans cells in the epidermis, Kupffer cells in the liver, and microglia cells in the central nervous system (CNS). APCs express both class I and class II MHC, which allow the recognition of the antigen by the TCR of $CD8^+$ T cells or $CD4^+$ T cells, respectively. Moreover, a different type of DCs was described, the so called plasmacytoid DCs. These cells were shown to produce high levels of type I IFN, and are thought to play important roles in antiviral host defense and autoimmunity[23]. Recent studies on DC differentiation have shown that both myeloid and common lymphoid progenitors can give rise to both conventional DCs and plasmacytoid DCs[24,25].

B cells

Adaptive humoral immunity is mediated by antibodies produced by plasma cells that develop from B cells under the direction of signals from T cells. B cells differentiate from hematopoietic stem cells in the bone marrow. It is there where their antigen receptors are assembled, in a RAG-mediated process, similar to the production of functional TCR[26]. B cells constitute around 15% of peripheral blood leucocytes. The immunoglobulin (Ig) (or antibody) production is an important aspect of the specificity of adaptive immunity. These molecules are composed by two heavy and two light chains. The amino-terminal portions of both heavy and light chains vary in amino-acid sequences from one antibody molecule to the other. These hypervariable sequences are brought together to form the

antigen-binding domain of the molecule. The carboxyl terminal regions of the heavy and light chains are constant in each subclass (isotype) of antibody. The heavy chain constant regions pair to form the Fc domain of the antibody, which is responsible for most of the effector functions of the Ig molecule, namely binding to Fc receptors and activating the complement system. Depending on the Fc region antibodies are classified as IgM, IgD, IgG, IgA, and IgE. Naïve B cells express IgM and IgD on their surface. As B cells mature, under the influence of $CD4^+$ T helper (Th) cells, T cell-derived cytokines induce isotype switch[27]. The same cell can then produce antibodies with different effector functions (different isotypes), but with the same antigen specificity (although the specificity can change due to the process of affinity maturation) [28]. IgA is usually found in mucosal environments (under the influence of TGF-β and IL-10)[29]. IgG subtypes are associated to different types of immune responses: IgG1 and IgG3 are found mainly in the context of allergic responses, under IL-10 influence (Th2 associated responses)[30], while IgG2 is most common in immune responses elicited following viral infection or in the course of autoimmunity, under the effects of IFN-γ and IL-1. IgE is also associated to immune responses targeting parasites or allergens, and the isotype switch occurs under the influence of IL-4 and IL-13 (Th2 associated)[31].

In addition, another contribution for B cell-specificity and variability derives from somatic hypermutations[32], taking place at the time of isotype switch. This process leads to the random introduction of mutations in the CDR domains involved in antigen recognition. Although random, coupled with selection of higher affinity mutants, leads to a progressive increase of the affinity to the antigen. It is this high affinity that provides the cell with a proliferative advantage in response to the antigen, and allows this antibody producing cell to dominate the pool of responding cells. Otherwise, if the somatic hypermutation causes a decreased affinity to the antigen, the cell loses important growth signals and dies.

Isotype switch and somatic mutations are strongly associated to the development of B- cell memory, which allows a quick and more efficient secondary response. The development of B cell memory requires B cell maturation, and this might be highly dependent on T cell help (through cytokine production). Memory B cells are also reported to be important players in the perpetuation of many allergic and autoimmune pathologies.

T cells

T cells develop in the thymus from common lymphoid progenitors coming from the bone marrow or fetal liver[33]. T cells are characterized for bearing a TCR, each one, with a single antigen-specificity. The process of TCR rearrangement and definition of T lineage commitment occurs in the thymus[34]. The thymus contains 3 compartments: the subcapsular zone, where recently arrived cells rapidly expand under the influence of IL-7 and begin to differentiate, proliferate and rearrange their TCR β chains. Then the cells move to the thymic cortex, where α-genes rearrange, potentially forming a functional mature αβ TCR (in the case of conventional T cells). Here, developing thymocytes are screened for their TCR affinity to MHC molecules (positive selection), which permits them to recognize antigen-MHC complexes. This happens through interactions between the developing lymphocyte and the specialized cortical epithelium[35]. If the TCR fails to bind its ligand with sufficient affinity, the thymocyte undergoes apoptosis and it is cleared by the thymical cortical macrophages[36]. The cells which express a TCR with sufficient affinity for an MHC peptide migrate to the medullary areas, where they differentiate into single positive $CD4^+$ and $CD8^+$ thymocytes. In the thymic medulla, cells are screened for potential autoreactivity (negative selection). This screening includes testing for reactivity towards an extensive array of tissue-specific proteins that are expressed by a population of thymic medullary epithelial cells, under the control of a gene called autoimmune regulator (AIRE)[37]. Cells that bind with high avidity to self-MHC/peptide are eliminated by means of apoptosis, ensuring that much of the autoreactive T-cell precursors are not allowed to mature (central tolerance). However, most, but not all, peripheral self-antigens are present in the thymus, and some immature self-reactive T cells never engage self-antigens, and migrate to the periphery as naïve “ignorant cells”[38]. Even though central tolerance contributes for the deletion of most potentially autoreactive T cells, still some autoreactive clones are found in the periphery of healthy individuals, but still kept under control. Some of the auto-reactive cells that exit the thymus express the transcription factor Foxp3 are endowed with immune regulatory function[39,40]. Fewer than 5% of the developing T cells survive positive and negative selection (Figure 1).

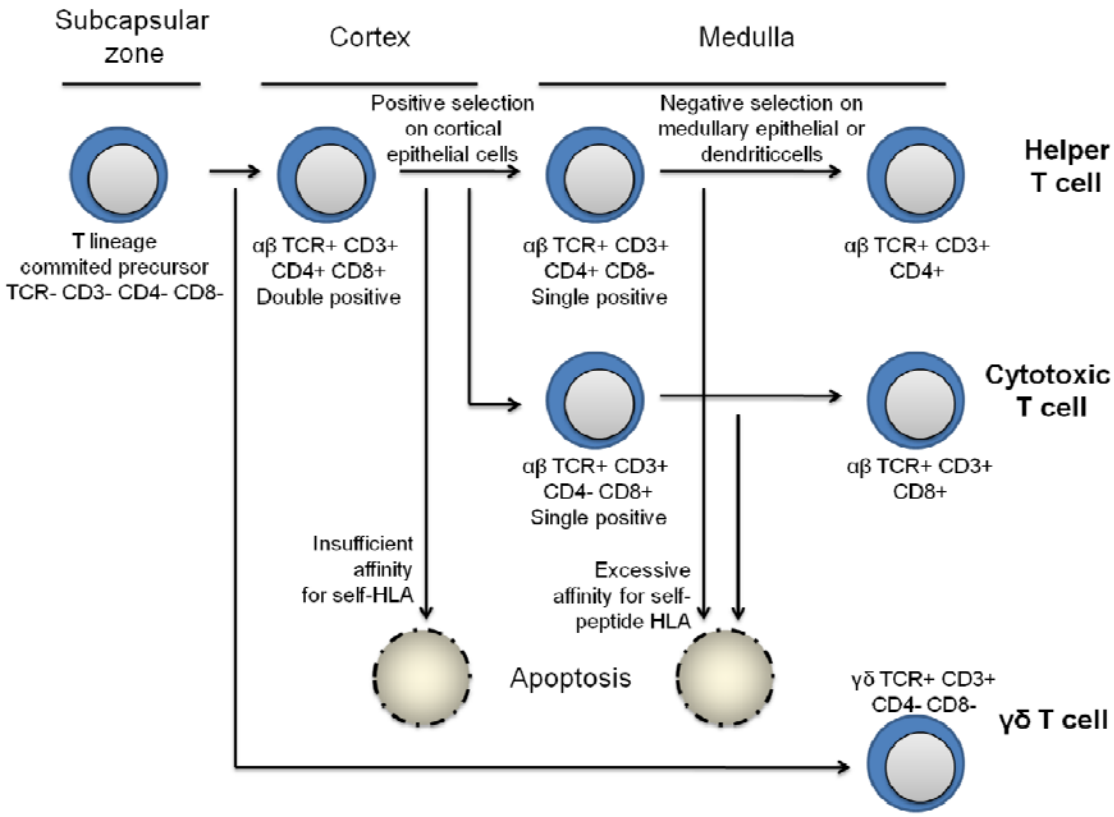


Figure 1 – Differentiation and maturation of T cells in the thymus – Hematopoietic stem cells, which do not express CD3, CD4 or CD8 but are commited to T cell differentiation, move from the bone marrow to the thymic subcapsular zone. There they begin rearrangement of the TCR genes. Once a productive TCRβ chain has been produced, they move to the thymic cortex, where TCRα chain rearrangement occurs and surface expression of the CD3, CD4 and CD8 proteins is induced. These CD4+ CD8+ (double-positive) cells are positively selected on cortical epithelial cells for their ability to recognize self class I protein, then it retains expression of CD8. If the cell recognizes a self class II protein, then it retains expression of CD4 and extinguishes expression of CD8. Selected CD4 or CD8 single-positive cells then move to the thymic medulla, where they are negatively selected on medullary epithelial cells to remove cells with excessive affinity for self-antigens presented in HLA molecules. Cells emerge from positive selection single positive for CD4 or CD8 expression and then are exported to the periphery. Cells that fail positive or negative selection are removed by apoptosis. A small fraction of cells differentiate to rearrange their TCR δ and γ chain, rather than α and β.

The antigen-specific TCR chains associate with invariant accessory chains that serve to transduce the signal when TCR binds to antigen-MHC complexes[41]. These accessory chains form the CD3 complex. Interaction of the TCR/CD3 complex with the antigen presented in a HLA molecule, provides only a partial signaling for T cell activation. Full activation requires the participation of co-stimulatory molecules such as CD28 on T cell that binds CD80 (B7.1) or CD86 (B7.2) on the APC[42], and a growing number of characterized costimulatory receptor:ligand molecules has been described, including

ICOS:ICOSL, CD40L:CD40 and OX40:OX40L (Figure 2). In fact interaction of peptide-MHC with the TCR without any co-stimulation, usually leads to an anergic state of prolonged T-cell non responsiveness[43]. When cells engage CD3, and their co-receptors and costimulatory molecules, it triggers a cascade of activation, involving phosphorylation of several proteins (for instance ZAP-70), leading ultimately to the activation of genes that control lymphocyte proliferation and differentiation. Other molecules have been described, namely cytotoxic T cell-associated antigen (CTLA-4), PD-1 and PDL-1, which inhibit these pathways of proliferation and effector functions, extinguishing T cell response[44].

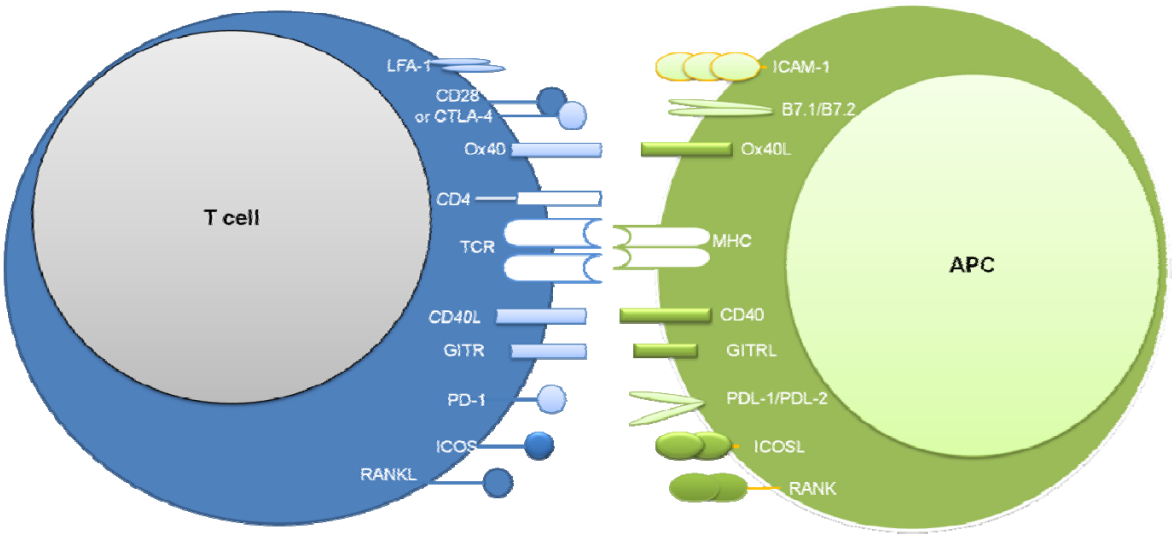


Figure 2 – Costimulatory molecules - Immune responses are triggered by activation of the T cell receptor with foreign antigen. Costimulatory molecules are important molecules to complete the crosstalk between the lymphocytes and the antigen presenting cell (APC). Each costimulatory molecule delivers different signals into the cell. The figure displays the most common costimulatory molecules, and the respective ligands.

The major class of T cells is defined by the surface expression of the αβ TCR (90 to 95%). This receptor has evolved primarily to recognize peptide antigens in a complex with class I or class II MHC proteins. αβ T cells differentiate into 2 major subsets: the $CD8^+$ T cells (30 to 40% of peripheral T cells) which are also called cytotoxic T cells, having a role in killing cells infected with intracellular microbes in a contact-dependent manner, activating apoptosis in the target cell; and the $CD4^+$ T cells (60 to 70% of peripheral T cells), that although able to deliver cytotoxic stimuli, have a major role in modulating cellular and humoral immune responses, through the production of different cytokines, and which will be extensively described below.

Then there are other minor subsets of T cells, the so called unconventional T cells: like γδ T cells and NKT cells. γδ T cells are a small subset of T cells (5-10% T cells), usually negative for CD4 and CD8, expressing a TCR with γ and δ chains. A portion of these cells is known to have origin in the thymus, but a major part seems to be generated in an extrathymic compartment, resulting in cells that largely populate the gastrointestinal tract[45]. Unlike αβ T cells, γδ T cells do not recognize antigens in the context of class I and class II MHC molecules, and their antigen recognition is still unclear[46]. However, these cells can be activated by small molecules like phospholipids and alkyl amines. These cells are thought to respond to mycobacterial antigens, and are known to play important cytotoxic roles against several tumour cell lines[47,48]. NKT cells express simultaneously αβ TCR and several NK markers, like CD56 or NK1.1 receptor. They recognize glycoplipid antigens presented by CD1d, a non-classical MHC molecule of the CD1 family. These cells are characterized by their ability to rapidly produce large amounts of different cytokines, like IFN-γ, IL-4, GM-CSF and TNF[49], and more recently were shown to produce IL-9[50] and IL-17[51], under specific environment conditions. Their role is still controversial, but they have been implicated in the pathogenesis of different immune mediated diseases[52].

1.1.3. CD4+ T cells

$CD4^+$ T cells are major mediators of both protective and pathogenic immune responses. They have the ability to produce highly specific antigen responses, with potent effector functions, involving the recruitment of other cell types. Moreover, they originate a subset with immunological memory, following the primary response. Change in the normal regulation of $CD4^+$ T cells can lead to serious damage to the host resulting in immune-mediated diseases, such as autoimmunity or allergy. Throughout the years several subsets (either involved in effector functions or regulatory mechanisms) have been described.

More than twenty years ago, Mosmann and Coffman proposed that T helper cells could be categorized in two distinct subsets, Th1 and Th2[53]. Th1 cells produce large amounts of IFN-γ, IL-2, IL-12 and TNF, whereas Th2 cells produce IL-4, IL-5, IL-13 and IL-25[54].The transcription factors associated to Th1 and Th2 type of responses are T-bet and GATA-3, respectively. Accordingly, Th1 cells induce immune responses of the delayed type hypersensitivity (DTH) and are very efficient in clearing intracellular pathogens, while Th2 cells are essential in promoting eosinophilic and humoral immune responses, and have a role in host defense against parasitic infections. Only recently, with the discovery of IL-17-producing $CD4^+$ T cells (Th17), the Th1 and Th2 dichotomy had to be revisited. IL-6 and TGF-β were shown to be important differentiation factors to this new T cell

lineage[55-57], and IL-23 was shown to be essential for its growth and maintenance[58]. ROR-γt was defined as the master transcription factor of Th17 cells[59]. Th17 play a role in host defense against certain extracellular pathogens, and funghi, and act by inducing pro-inflammatory cytokines like IL-6 and TNF production, as well as driving neutrophil recruitment and activation, and tissue damage. In addition to these 3 subsets of effector T cells, another functional type has been recently described, these were named Th9[60,61] cells as a consequence of their ability to produce high amounts of IL-9, a potent mast cell growth factor and mediator of helminthic immunity. The transcription factor associated to this subset was described to be PU-1[62]. Their differentiation was shown to be driven by TGF-β and IL-4[60,61]. Last, but probably not least, emerging evidence suggests the existence of Th22 cells, a brand new subset, characterized by the production of IL-22 and TNF, but not IFN-γ, IL-4 or IL-17. It was, until now, found to be involved in epidermal immunity and remodeling, being detected within the epidermal layer in inflammatory skin diseases[63] (Figure 3).

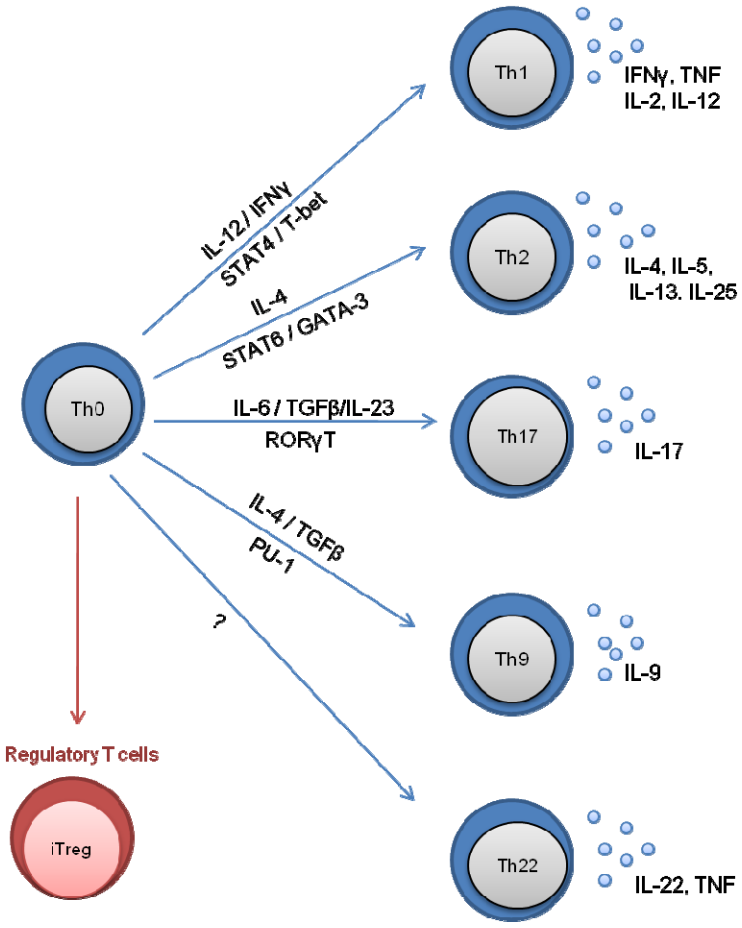


Figure 3 – Differentiation of naive CD4+ T cell lineage into effector T cells – The transcription factors and cytokines required to promote the differentiation of the naive T cells into the distinct lineages are shown. Th subsets are defined according to their production of lineage –indicating cytokines and function

Regulatory T cells (Tregs) are another distinct lineage of $CD4^+$ T cells, and have been well characterized as specialized cells in immune suppression[64-66]. Although different T cell subsets with immune suppressive function have been described, the best characterized are defined by the expression of Foxp3, a transcription factor required for their function and stability[65,67-70]. Other markers (surface molecules), have also been associated to this subpopulation, like CTLA-4, glucocorticoid-induced TNF related protein (GITR) and CD25. The other Tregs which do not express Foxp3 transcription factor, namely Tr1 and Th3 cells[71,72], are mainly characterized, by IL-10 or TGF-β expression, respectively. $Foxp3^+$ Tregs can be natural Tregs (nTregs), coming originally commited as regulatory cells from the thymus[73], or induced in the periphery[74] from $Foxp3^-$ precursors (iTreg).

This phenomenon of inducing regulatory T cells in the periphery brought a whole new perspective about $CD4^+$ T cell commitment and plasticity, suggesting the capacity to redirect their functional programs, thus affecting the balance between Tregs and cytokine-producing effector T cells. Tight regulation of effector T cell responses is required for effective control of infections and avoidance of autoimmune and immunopathological diseases. Aberrant Th1 and Th17 cell responses play critical roles in organ-specific autoimmunity[75-77], whereas Th2 cells are culprits in allergy and asthma[78]. Treg cells play essential roles in the maintenance of immune homeostasis, regulating these effector T cell responses and thus preventing their potentially pathogenic effects through a variety of mechanisms (discussed below). It is now generally assumed that cytokines which induce proinflammatory T helper type differentiation, such as IL-6, antagonize Foxp3 induction in the periphery[79,80]. Th17 and $Foxp3^+$ Tregs share a cytokine which is crucial for the differentiation of both types of lineages – TGF-β[55-57,74]. However, it is the rest of the cytokine environment which dictates which population will differentiate. In the presence of IL-6, IL-21 or IL-23, Th17 will prevail[57], as IL-6 will inhibit Foxp3 expression. In contrast, if the cytokine milieu is anti-inflammatory, Foxp3 regulatory cells will more easily differentiate over Th17. Moreover, Foxp3 expressing T cells can be converted to IL-17-expressing cells in a proinflammatory cytokine environment[80,81], however, one study suggest that unlike nTreg cells, iTreg induced by TGF-β and IL-2 are resistant to Th17 conversion by IL-6[82]. All these evidences suggest that $CD4^+$ T cells are highly interconvertable and may assume different roles depending on the conditions they are exposed to. It is now emerging that every adaptive immune response involves

recruitment and activation of not only the before mentioned effector T cells, but also Tregs, and that the balance between these populations is critical for the proper control of the quality and magnitude of adaptive immune responses, and for establishing or breaking tolerance to self and non-self antigens.

1.1.4.Regulatory T cells

Back in the 80s and early 90s, some evidence arose for participation of $CD4^+$ T cells in preventing damaging immune reactions[83-87]. At the time, no marker was available to distinguish the putative regulatory T cells within $CD4^+$ population. Low CD45RB in mice (CD45RC in rats), and later, CD25 were the first markers to characterize this specific subset[88], although far from perfect, as they were upregulated in activated T cells. In 1995, Sakaguchi showed that depletion of a minor population of $CD4^+$ T cells expressing CD25 from a population of adult $CD4^+$ T cells, led to the generation of a spectrum of autoimmune diseases when transferred to an immunocompromised recipient[88]. Moreover, co-transfer of $CD25^+$ T cells prevented autoimmunity. A "natural" thymus-derived regulatory subset was then defined based on CD25 expression in a resting immune system, a population that would also be found in humans[89]. The finding that $CD4^+$ $CD25^+$ T cells could suppress T cell proliferation *in vitro*[90] led to the adoption of this assay to test functional Tregs in lymphocyte populations. Later, the forkhead box transcription factor Foxp3 was identified as an essential transcription factor in $CD4^+$ regulatory T cells[65-67], a major breakthrough, to establish regulatory T cells as a bonafide subset. In addition, patients with IPEX syndrome, a disease with diverse immunopathologies, carried mutations in the gene for the transcription factor Foxp3 and lacked Tregs[91]. Moreover, when $Foxp3^+$ Tregs are depleted in an adult mouse, fatal multi-organ autoimmunity results, and the phenotype of this disease is virtually indistinguishable from the IPEX syndrome in humans (equivalent to Scurfy phenotype in mice)[92-94]. Moreover, murine studies clearly showed the implication of Foxp3 as the main transcription factor required for $CD4^+$ T cells to become regulatory[65,67]. Importantly, given the central role Foxp3 plays in maintaining Treg cell transcriptional program and cellular phenotype[95,96], maintenance of Foxp3 expression is central to Treg cell lineage stability. In the last few years, it has become clear that $Foxp3^+$ regulatory T cells can be induced outside the thymus, through signals mediated via the TCR, together with other extrinsic factors, like TGF-β or IL-2 [74,97,98].

Natural Foxp3+ Treg

Naturally occurring Tregs are generated in the thymus. This process requires TCR triggering in the presence of costimulation[99,100]. However TGF-β and IL-2 are dispensable[101,102]. Controversially, recent studies showed that CD4- CD8- double negative (DN) human thymic population express Foxp3 in the absence of TCR, suggesting that Foxp3 can develop in the thymus from the DN population[103]. Although still diverse, the repertoire of nTregs may be skewed towards self-tissue antigens[104]. Numerous studies have demonstrated that nTregs are generated in the thymus through MHC class II-dependent TCR interactions, with high avidity towards thymic self-peptide ligands, and this highly self-reactive T cells would be recruited to the T cell lineage in the course of T cell selection[39,40,105-107], although additional selection mechanisms may take place[107]. Notably the expression of AIRE, which is responsible for the negative selection of auto-reactive T cell clones, seems to be required for the "positive selection" of Tregs[108]. Some reports suggest that Foxp3 expression is dispensable for Tregs thymic selection process, but essential to stabilize the regulatory phenotype of these cells when in the peripheral immune compartment[95,96]. They have also shown that loss of Foxp3 in Tregs results in effector-like T cells, with increased production of Th1 cytokines and IL-17, and an increased potential for tissue infiltration.

Once in the periphery, natural occurring Tregs represent around 6-10% fraction of the overall $CD4^+$ T cells, and in order to be maintained they need continuous TCR triggering and co-stimulation in the presence of IL-2[109-111]. So IL-2 is important for nTregs pool maintenance in the periphery[112].

Foxp3+ Treg induction in the periphery

Concerning iTregs, increasing evidence points out to a variety of conditions which allow iTregs generation in the periphery[74,113,114] (Figure 4). Importantly, when the key role for Foxp3 in Treg biology was demonstrated, non regulatory T cells were shown to be able to acquire Foxp3, and consequently, regulatory function[113,115,116]. First *in vitro* evidences for Treg conversion were shown to be driven by TGF-β in the presence of TCR triggering and costimulation[74]. Additional studies supporting this observation showed that conversion of naïve T cells is facilitated by a suboptimal TCR signal or by a combination of strong TCR signal with high amounts of TGF-β [74,114,117,118]. Early evidences for *in vivo* peripheral conversion were originated from adoptive transfer experiments in which polyclonal $CD4^+$ $CD25^-$ naïve T cells were injected into lymphopenic mice or mice containing a monoclonal T cell repertoire devoid of nTregs[116,119]. In such conditions, homeostatic proliferation of the donor cells took place, and part of the donor population

became $CD25^{+}CTLA\text{-}4^{+}GITR^{+}$ and acquired Foxp3 expression and suppressive activity. Moreover, when congenically marked $CD4^{+}$ $CD25^{-}$ T cells were transferred to WT hosts, 10% of those converted into Foxp3 expressing $CD4^{+}$ $CD25^{+}$ T cells, within 6 weeks, and co-stimulation through B7 family molecules was essential[120]. CD28 co-stimulatory signals have an essential cell intrinsic role in the differentiation of Tregs, supported by the decrease of Treg levels in CD28-deficient and CD80/86-deficient mice[100,121]. iTregs induction by foreign antigens has been described by several groups. Von Bohemer and colleagues have shown that continuous administration of low dose antigen without inflammatory stimuli induced the conversion of $CD4^{+}$ $CD25^{-}$ into $CD4^{+}$ $CD25^{+}$ Tregs[113]. Lafaille described the induction of OVA-specific $Foxp3^{+}$ regulatory T cell in the mesenteric lymph nodes of mice exposed orally to this antigen, the so called oral tolerance[122]. Monoclonal antibodies have also been widely used for tolerance induction, through conversion of regulatory T cells. Waldmann and coworkers have shown that, in the presence of a non-depleting anti-CD4 monoclonal antibody, mice became tolerant to allogenic transplants, through the conversion of naive CD4+ T cells into $Foxp3^{+}$ $CD4^{+}$ Treg cells[115]. TGF-β role was shown to be necessary for this induction of peripheral tolerance[115,123]. Also, treatment of non-obese diabetic (NOD) mice with CD3-specific antibody induces a population of Treg cells that suppresses diabetes[124]. Antigen presentation by immature or tolerogenic DCs, or antigen that targets DCs through DEC-205, might provide the right cytokine milieu that favors Treg cells conversion[114,125]. Additionally, $CD103^{+}$ DCs, which are present in the gut draining mesenteric lymph nodes (LNs) are able to induce Foxp3 expression in naïve $CD4^{+}$ T cells, through production of TGF-β and retinoic acid[126,127].Thus, it is clear that naïve T cells selected as Foxp3- in the thymus, have full potential to become Foxp3+ T cells *in vivo* and *in vitro*.

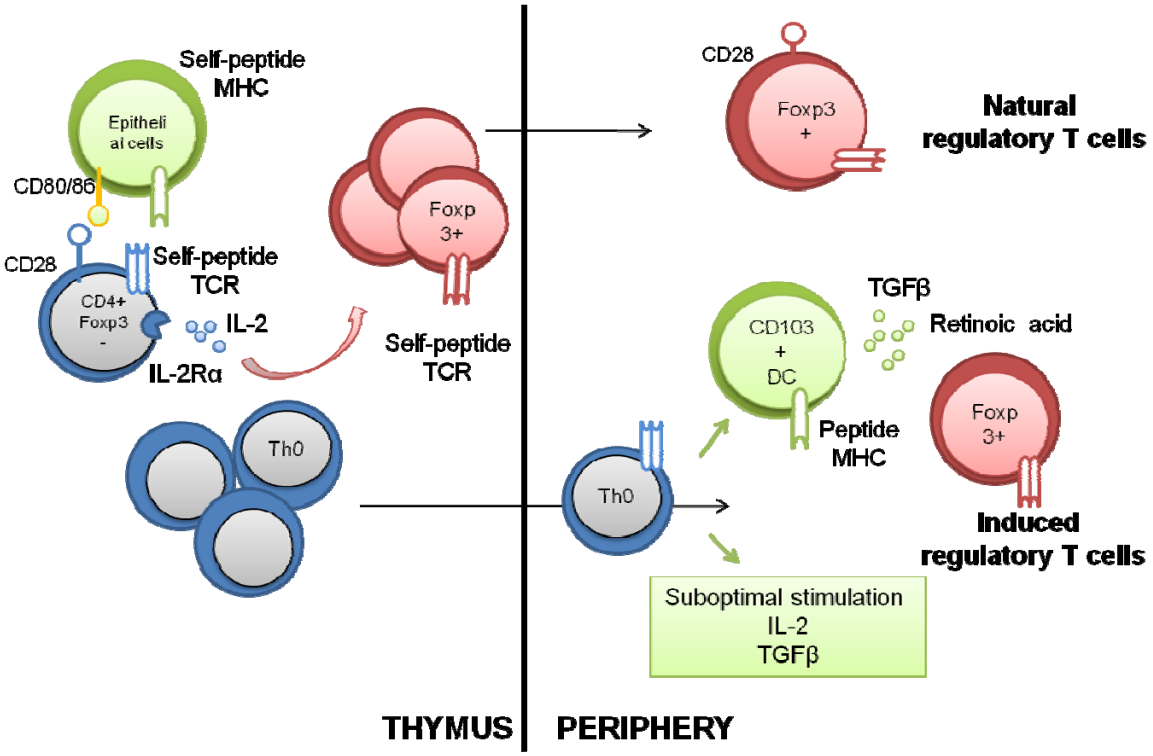


Figure 4 – Differentiation of thymic and Induced Tregs – Most Foxp3+ thymic Treg cells, differentiate from Foxp3-negative CD4 single positive thymocytes. The process of Treg cell differentiation as defined by induction of Foxp3 requires: increased strength of TCR stimulation by self-peptide-MHC complexes presented by thymic epithelial cells (TECs) or DCs, CD28 signaling induced by CD80 and CD86 ligand expressed on APCs, and high-affinity IL-2 receptor and other γchain-cytokine receptor signaling. Treg cell homeostasis is dependent on exocrine IL-2 produced by effector T cells. Foxp3+ Tregs can also be induced from peripheral naive CD4+ T cells. Conditions favoring the peripheral induction of Foxp3 include chronic low dose antigen stimulation under tolerizing conditions. iTreg cells are likely prominent in the gut-associated lymphoid tissue where chronic exposure to food, commensal, or environmental antigens probably facilitates their generation. Suboptimal costimulation is critical for the differentiationof iTreg cells with a particularly important role for the immunomodulatory cytokine TGFβ. Additionally, IL-2 and the vitamin A metabolite retinoic acid facilitate induction of Foxp3 in peripheral Foxp3 CD4 T cells. CD103+ DCs, which produce retinoic acid and TGFβ, are potent inducers of Foxp3 expression in activated T cells. These DCs are present in high numbers in the gut where they probably limit immune inflammation through the generation of iTregs.

Mechanisms of action of regulatory T cells

Although nTregs and iTregs commonly express Foxp3, presently it is not clear whether these two modes of Treg cell differentiation serve different biological needs or have partially or fully redundant functions. It is also unclear whether mechanistic requirements for Treg cell generation in the thymus and in the periphery are distinct. It has been reported that Foxp3 Treg cells exert suppression through cell contact-dependent mechanisms, as well as by soluble mediators (Figure 5).

Cell contact mechanisms may lead to functional modulation mediated by molecules such as GITR, CTLA-4, CD39, CD73, and lymphocyte activation gene (LAG)-3, or killing of APCs and responder T cells through granzyme B and perforin[128,129]. Soluble factors involved in suppression can be immunosuppressive cytokines, such as IL-10, TGF-β, IL-35 and galectin-1, or deprivation of cytokines involved in the expansion and/or survival of responder T cells, for instance IL-2[129,130]. It is not clear, whether there is a main mechanism that is common for Treg suppression, in humans and in mice. The main candidate for this core mechanism in Tregs regulation is CTLA-4 –dependent suppression, as several studies have shown an impairment in Tregs function when this molecule is blocked, causing IBD in healthy mice[131], exacerbating diabetes in NOD mice[132], and the absence of CTLA-4 lead to a variety of autoimmune diseases and IgE hyperproduction, similarly to what happens in Foxp3-deficient mice[133]. Foxp3 natural Tregs constitutively express high amounts of CTLA-4[134]. Also, in humans, terminally differentiated $Foxp3^{hi}$ $CD25^{hi}$ $CD4^{+}$ T cells, which are highly suppressive in vitro, constitutively express CTLA-4[135]. Importantly, Foxp3 upregulates the expression of CTLA-4, indicating that Tregs transcription factor Foxp3 may sustain the high expression of CTLA-4 in regulatory cells[136,137]. The role for CTLA-4 molecule may underlie on its ability to downmodulate APC function, inhibiting upregulation of CD80 and/or CD86[138]. Besides, CTLA-4 ligation to CD80 or CD86 might activate a pathway leading to the production of the immunosuppressive kynurenine, or activating immune regulating Foxo3, which inhibits cytokine production by DCs[139]. Collectively, these studies suggest that CTLA-4 expression by Foxp3 regulatory T cells is essential for their ability to sustain self tolerance and immune homeostasis. Other suppressive mechanisms may occur depending on the environment, biological context and immune response. For instance, IL-10 producing cells are more abundant in lamina propria[140,141], perforin or granzyme expressing Tregs are predominant in tumor environment[142]. Moreover regulatory T cell can functionally differentiate to specifically inhibit Th1, Th2 or Th17, whose transcription factors may affect chemokine receptors expression that will facilitate Treg cell migration to the inflammation site[143,144].

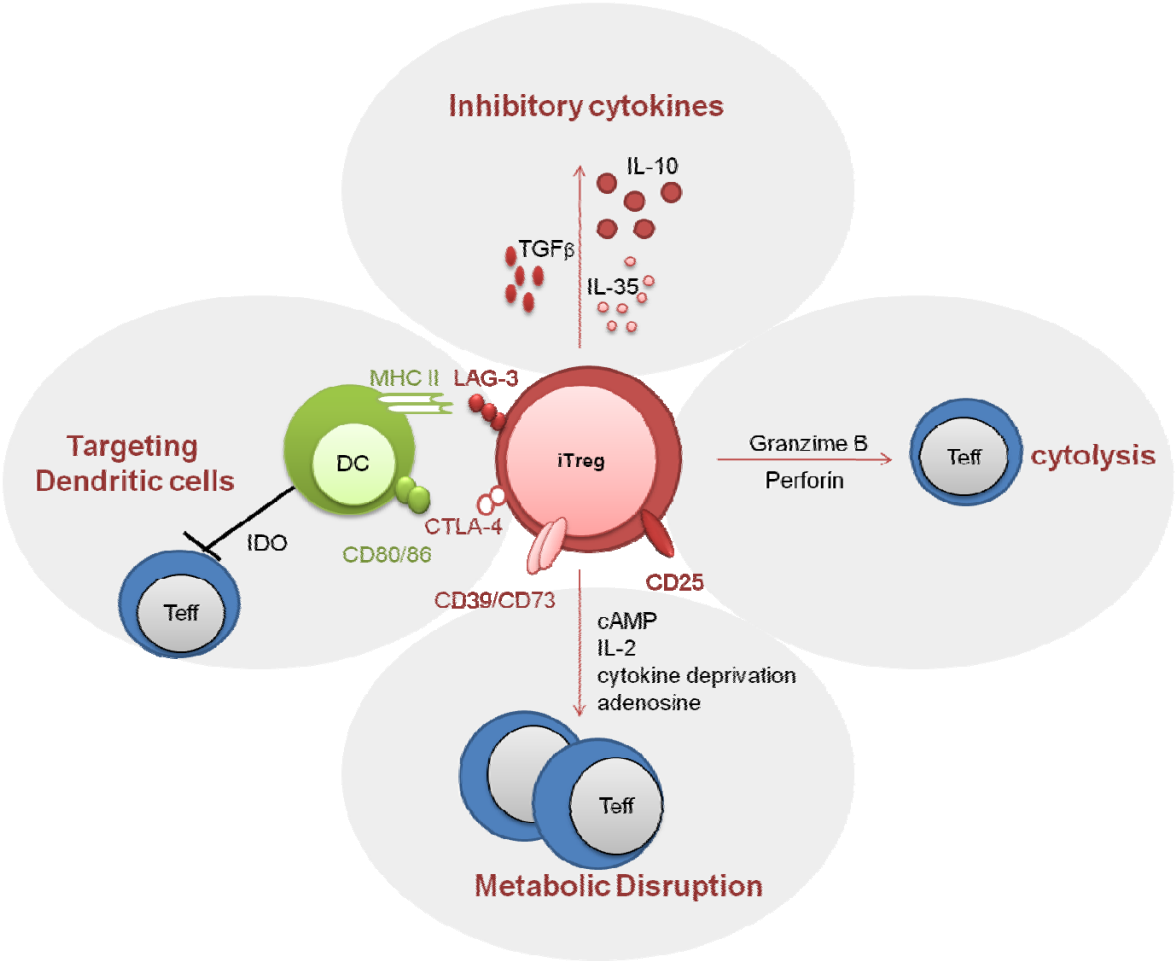


Figure 5 – Basic mechanisms used by Tregs – The various Treg cell mechanisms can be arranged into four main groups, based on their modes of action: 1) Inhibitory cytokines – include IL-10, IL-35 and TGF-β; 2) Cytolisis- includes granzyme B-dependent and perforin-dependent killing mechanims; 3) metabolic disruption – includes high affinity IL-2Rα (CD25)-dependent cytokine deprivation-mediated apoptosis, cyclic AMP-mediated inhibition. And CD39 and/or CD73-generated, adenosine purinergic adenosine receptor (A2A)-mediated immunosuppression; 4) Targeting DCs- includes mechanisms that modulate DC maturation and/or function such as LAG-3, also known as CD223 - MHC class II-mediated suppression of DC maturation, and CTLA-4 - CD80/86-mediated induction of indoleamine 2,3-dyoxigenase (IDO), which is an immunosuppressive molecule by DCs.

As a final remark, nTregs and iTreg cells play essential roles in immune tolerance and in the control of severe chronic allergic inflammation. Although iTregs seem tailored to respond to foreign antigens and neoantigens (such as tumor antigens), it is likely that they are also generated in response to self-antigens or allergens, and synergize with nTreg cells to fight autoimmune or allergic inflammations.

Foxp3 Stability

Foxp3, the characteristic transcription factor of regulatory T cell lineage, was reported to be essential for the maintenance of Treg suppressive function. It was suggested that Foxp3 may control its own expression through a positive feedback loop[96,145], even though other studies suggest that, at inflammatory sites, Tregs show a lower expression of Foxp3, which can lead to a higher susceptibility to autoimmunity[146,147]. Supporting these observations is the fact that both IL-1 and IL-6 proinflammatory cytokines inhibit Foxp3 expression[80]. Moreover, peripheral Tregs are usually unstable in a lymphopenic host, and can convert to follicular helper T cells[148,149]. Altogether, these data suggest that Foxp3 may be unstable under certain conditions. Moreover, a recent study using a Foxp3 reporter lineage marker, shows that indeed a percentage of the Foxp3 expressing T cells lose its expression, and this loss increases in an autoimmune context. So, it appears that even in normal conditions, Foxp3 expression is regulated according to physiological conditions. As impressive as it may sound, the regulatory T cells that loose Foxp3 expression and consequently their suppressive functions, can even become effector T cells, and start to produce pro-inflammatory cytokines like IFN-γ and IL-17[150]. These cells that loose Foxp3 are characterized by the expression of high levels of CD127, a memory T cell marker[96,151], and were shown to be able to transfer autoimmune diabetes[150].

Overall, in a proinflammatory environment, like in the case of autoimmune and allergic diseases, pathogenesis will easily prevail, because surrounding inflammatory conditions inhibit Foxp3 stability and consequently the maintenance of Tregs suppressive function. Moreover, if Treg cells bear mostly self-specific TCRs, they can become a major threat when subjected to lose Foxp3 expression and turn effector T cells. Ideally, to avoid an imbalance between Tregs and effector T cells, and consequent loss of tolerance, the triggering of pro-inflammatory signals should be impaired, for instance through blocking T helper cells activation and proliferation.

1.2. Immune Tolerance

A major challenge in immunology and medicine is to determine how unresponsiveness of the adaptive immune system to self-antigens, allergens or commensal bacteria is maintained – this feature is called immune tolerance. There are several mechanisms that help the immune system to maintain tolerance. The process of generating diversity within the adaptive immune system needs a quality control in order to prevent the generation of functional lymphocytes bearing self-antigen specific receptors[152] – a

mechanism which occurs in the thymus, called central tolerance. As discussed above, the thymus plays an important role in the maintenance of tolerance and although its size diminishes with age, it has been shown that the thymus remains functional throughout adult life. As not all self-antigens are expressed in the thymus, other mechanisms exist in the peripheral immune system to maintain a safe T cell repertoire. It has been shown that during thymic selection, when the TCR interaction of developing thymocytes is somewhere in between the endpoints for negative and positive selection, instead of being deleted, these cell will acquire Foxp3 expression and a regulatory phenotype once exported to the periphery. Suppression seems to be the central mechanism mediating peripheral tolerance, and $Foxp3^+$ regulatory T cells play a pivotal role. These were shown to be major mediators in the maintenance of immunological tolerance and immune homeostasis, through active suppression of pathological and physiological immune responses[153]. The greatest evidence for Tregs crucial role mediating self-tolerance, is the fact that mutations in the Foxp3 gene lead to a deficiency in Treg cells and originate severe autoimmune manifestations (IPEX syndrome). Consistent with the human pathology, Tregs depletion in wild type mice, leads to the development of autoimmune disease, while their reconstitution prevents it[88]. Suppressive T cells important role had been evidenced several years before this observation, when spontaneous murine autoimmune ovarian disease was induced in day 3 thymectomized mice in 1969, and spontaneous autoimmune thyroiditis and diabetes were induced in adult-thymectomized mice in 1976, and in both cases, disease was prevented by adoptive transfer of total spleen cells from normal adult donors[154,155]. Taken together, these results show that the thymus is constantly producing nTregs which will be essential for tolerance maintenance. Besides Foxp3 expressing Tregs, Th3 and Tr1 cells are also important in immune tolerance maintenance, mainly in environments like the gut, which is populated with several commensal microorganisms, and where this type of cells is prevalent.

Tregs maintain tolerance

The important role for regulatory cells in the maintenance of immune tolerance is now clear, and is based in several crucial observations: the ability of Foxp3 expressing Tregs to inhibit the development of autoimmune disease caused by Treg depletion[88]; *in vitro* culture Tregs are able to suppress the proliferation of antigen-stimulated naïve T cells[90,156]; and normal naïve T cells that are forced to express Foxp3 become regulatory cells, with *in vivo* and *in vitro* suppressive functions[65,67].

The apparent distinction between central and peripheral tolerance mechanisms may be less clear, because naturally occurring Tregs appear to be selected in the thymus for self-antigen/MHC expressed by thymic epithelial cells[39,40]. This selection occurs at affinities that should lead to deletion, and therefore negative selection of Tregs. It is not known why some thymocytes escape deletion (negative selection) and differentiate into Treg cells, also the antigen specificity of Treg cells in normal (non transgenic) animals is not known. Presumably the regulatory cells in normal animals are polyclonal populations that recognize a diversity of self antigens, but it is unclear if they are biased towards recognition of a particular type or subset of self-antigens. Some studies suggest that for the maintenance of self-tolerance, the constant exposure to the self-antigen is required, and neonatal exposure is not a requisite[157]. Moreover, the majority of studies have shown that antigen-specific Treg cells are more potent suppressing the induction of autoimmune disease than polyclonal populations[158]. However several studies have also shown that polyclonal Tregs are able to suppress independently of its specificity[159,160]. Setoguchi et al identified a small proportion of polyclonal Treg cells with high regulatory capacity which undergo IL-2-dependent proliferation in the LNs[161]. Although suppressive activity of Tregs requires their prior activation through their TCR, once activated Treg cells suppress in an antigen-nonspecific manner. Thus, Treg with one antigen specificity can suppress effector cells with many other antigen specificities – bystander suppression. Moreover, transplantation studies have shown that Tregs can display a phenomenon called “linked suppression”, where they can be activated in an antigen specific manner, and subsequently suppress responses to unrelated antigens presented by the same cells[162]. In addition, the phenomenon of infectious tolerance is proposed on the basis of in vivo transfer studies in which one population of suppressor T cells creates a regulatory milieu that promotes the outgrowth of a new population of Treg cells with antigen specificities distinct from those of the original population[163]. Furthermore, new antigen specificities can be acquired by adaptive Treg cell populations as long as the new antigen is present in the same tissue that the antigen recognized by the original Treg cell was[86]. Thus through the process of bystander suppression and infectious tolerance, Treg cells effectively establish a state of dominant and stable tolerance.

An important question concerning Tregs mediated suppression *in vivo* relies on their homing, and whether their suppressive activity occurs on the secondary lymphoid organs, locally at the site of inflammation, or both. Studies done in the IBD model, where Tregs are able to control disease until the 4th week after its onset, have shown that Tregs accumulate in the mesenteric LNs as well as in the colonic lamina propria, being in direct contact with DCs and effector cells[164]. These findings suggest that regulation of an active

immune response occurs in both, the draining LNS and at the site of inflammation. Other studies in autoimmune diseases (such as EAE and diabetes), have equally shown the control of the disease through regulatory cells suppression at a local level, for instance in diabetes they are found preferentially in pancreatic lymph nodes[165] as well as pancreas islets[166], and in EAE in the cervical lymph nodes and CNS[167-169]. Overall, it is clear that the site of Treg cell action is certainly not limited to lymphoid organs. In many situations their ability to migrate to and remain in inflamed tissues is important for their function *in vivo*.

Tregs versatility and adaptability makes them "true masters of immune regulation" and central players in the maintenance of peripheral tolerance in non-inflammatory conditions, having an essential role in preventing spontaneous autoimmunity, as well as a broad spectrum of immune responses, either against tumor antigens[170], allergens[91], pathogenic or commensal microbes[171], allogenic organ transplants[172] and the fetus during pregnancy[173].

Activation Induced Cell Death

T cell apoptosis is believed to be an important process to maintain homeostasis and self-tolerance in the immune system. For example thymocytes that fail to rearrange their TCR will be neglected[174], and those that recognize self-antigens will be eliminated by apoptosis, a process called negative selection [175]. In peripheral T cells, a form of apoptosis induced by repeated TCR stimulation, known as activation-induced cell death (AICD), may be responsible for the peripheral deletion of auto-reactive T cells[176]. AICD results from the interaction between Fas and Fas ligand (FasL), and activated cells expressing both Fas and FasL are killed either by themselves or by interacting with each other[177] . Thus, Fas-mediated AICD is an important mechanism for maintenance of tolerance to self-antigens. This is illustrated by the autoimmune diseases that develop in mice and humans with inherited defects in Fas or FasL [178]. However AICD can also be Fas independent. When transplantation tolerance is induced following co-stimulation blockade, AICD is essential for the prevention of graft rejection, but the mechanism of AICD is Fas-independent[179].

1.2.1. Tolerance Breakdown

Assuming that multiple mechanisms contribute to the maintenance of immune tolerance to various degrees, at different levels, and in complementary manners, a crucial question would be which of these, can lead to development of immune mediated pathologies such as autoimmunity and allergy. Peripheral self-tolerance and immune homeostasis are maintained, at least in part, by the balance between Treg and effector T cells[153] (Figure 6). Tregs deficiency or dysfunction produces a variety of T cell mediated autoimmune diseases, allergy and immunopathology. For instance, anomaly of naturally arising Tregs can be a primary cause of autoimmune or inflammatory disease in humans[180]. Polymorphisms of certain T cell genes have been associated to autoimmune disease susceptibility, for instance CTLA-4 and IL-2 genes, which were shown to lead to higher susceptibility to type 1 diabetes (T1D), and other organ-specific autoimmune diseases in humans[181]. Moreover, other hypothesis have been proposed: genetic defects primarily affecting $CD4^+$ T cells development or function, microbial infections who mimic self proteins, or affect the balance between Tregs and effector T cells, inefficient thymic clonal deletion of self-reactive cells in neonatal period, altered thymic homeostasis that might elicit homeostatic proliferation of certain self-reactive clones, are all possible triggers for autoimmunity or other T-cell mediated diseases.

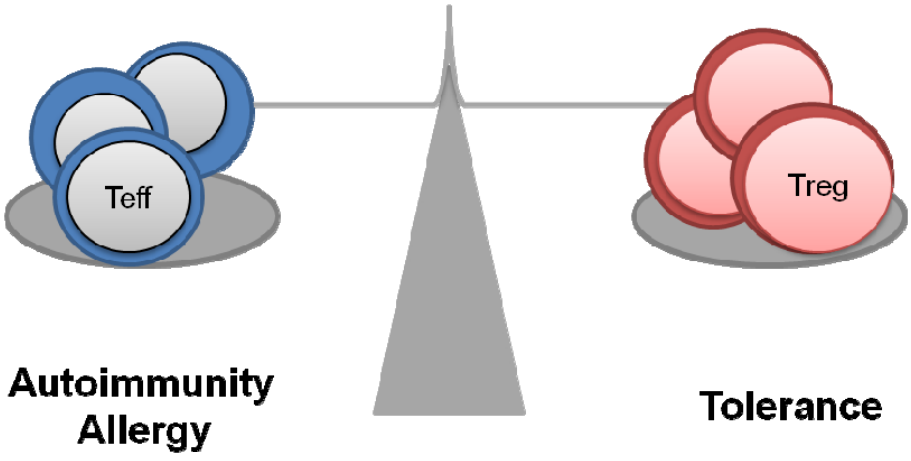


Figure 6 - Balance between effector T cells and regulatory T cells – Tregs have been shown to have a crucial role in the control of autoimmune and allergic diseases. Several studies have shown that patients with autoimmune and allergic pathologies have defective Treg levels or suppressive function. Therefore, there is

an important balance between effector and Treg cells, which determine the outcome of immune mediated pathologies such as autoimmunity and allergy, or tolerance.

Autoimmunity

Autoimmune diseases are a major cause of morbidity and mortality in the industrialized world, affecting 3-8% of the population. Autoimmunity is the failure of the immune system to maintain tolerance against 'self', a process that involves many different molecules and yet poorly understood mechanisms. There are several evidences which support three major hypothesis for the breakdown of Treg control: firstly Treg numbers are reduced and or dysfunctional because of inherent deficiencies in autoimmune susceptible individuals[180,182]; secondly Treg suppressive function is inhibited, diverted or converted by the chronic inflammation that characterizes autoimmunity[80,148,150]; and/or thirdly, self-reactive effector T cells become unusually aggressive and are refractory to regulation by otherwise functional Tregs, because they either overwhelm regulatory control or express molecules that render them resistant[183,184]. Genetic and environmental factors are also known to contribute to the prevalence of autoimmunity[181,185]. The spectrum of autoimmune diseases includes a large variety of diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS), T1D, Crohn's disease, between others, displaying different clinical features. However, despite the clinical differences there are as well, many pathogenic overlaps. Th1 and Th17 effector cells are now known to be major players in the pathogenesis of autoimmune diseases, and consequently, all the cytokines related to these subsets are equally involved.

In MS, for instance, the immune system attacks olygodendrocyte cells (ODCs) composing the myelin sheath which insulates neuronal axons. This inflammatory demyelinating disease interrupts electrical signaling and nerve impulses throughout the brain and spinal cord, leading to impaired motor movements and paralysis. EAE, an animal model for MS, is also a demyelinating autoimmune disease[38]. EAE as a model of MS is one of many important tools essential to the study of the pathophysiology and immunology underlying autoimmune diseases. The idea that all types of T cells were simultaneously required for autoimmunity development was refuted by the finding that MBP-specific T-cell clones could transfer EAE to nude mice[186]. In 1994 Lafaille *et al* showed that EAE could occur in the absence of B cells and antibodies as well[187]. In TCR transgenic mice, where the frequency of MBP-specific cells is increased, EAE may develop spontaneously[38]. In fact, TR- (RAG-1-/- background) mice, a MBP-specific TCR transgenic

mouse model for MS develop 100% EAE when no other lymphocytes are present, but in the presence of a normal immune system, only 14% of these mice develop disease[187]. So, if the frequency of auto-reactive T cells is high, antigen-adjuvant immunization is not needed to activate T cells. It appears that when a higher percentage of $CD4^+$ T cell population is antigen-specific, the chances for their activation are dramatically increased[187].

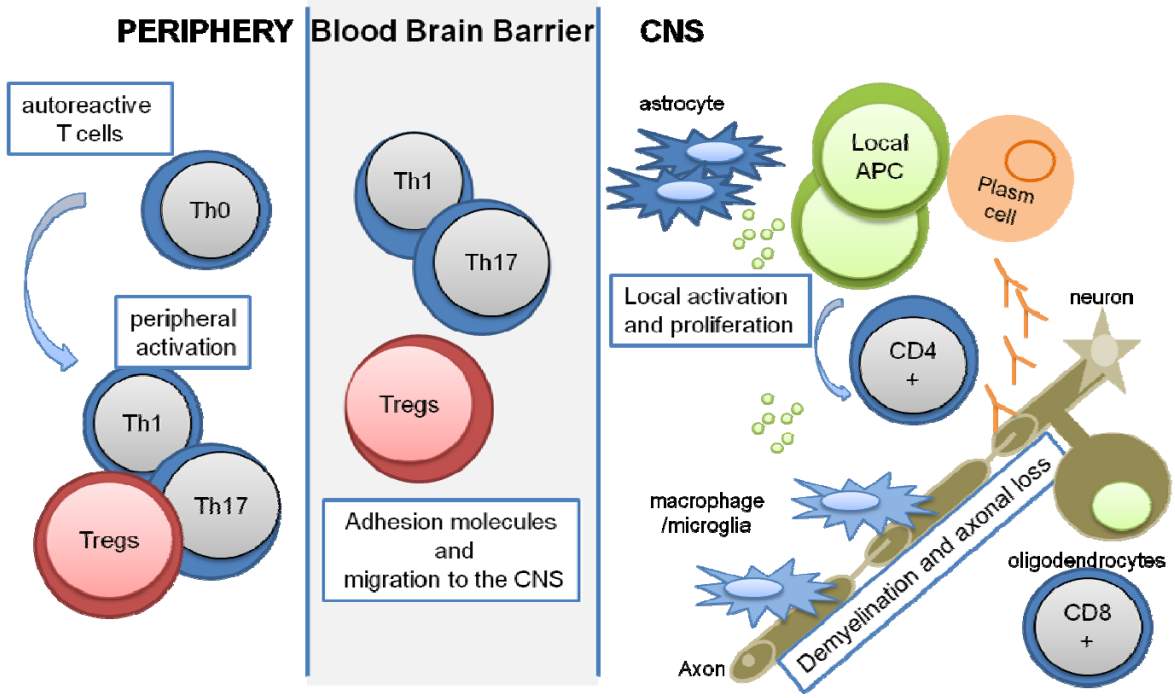


Figure 7 - Immunopathogenesis of multiple sclerosis – when predisposed individual's immune system encounters infectious agents, immune responses crossreactive with self-proteins occur in the peripheral lymphoid immune system. Activated antigen-specific T cells and B cells cross the blood-brain barrier and target self antigens expressed by ODCs and neurons. In concert with the innate immune response in the CNS, T and B cells cause inflammatory damage. Susceptibility of ODCs and neurons to inflammatory damage, and the capacity for CNS repair and reorganization, determines the extent and functional consequences of the inflammatory damage.

A number of immune and CNS cell types are involved in lesion development and repair. T cells, B cells and macrophages infiltrate the lesion. CD4+ T cells are located in the perivascular cuff. These cells become reactivated by antigens presented on DCs and microglial cells, and locally release cytokines and inflammatory mediators, thereby attracting macrophages to the lesions. CD8+ T cells infiltrate the parenchyma and, as well as secreting inflammatory mediators, they directly attack cells expressing HLA I such as neurons and ODCs. B cells are predominantly found in perivascular spaces and meninges, where they release IgG antibodies. These antibodies bind to proteins expressed on the surface of ODCs and neurons. Bound antibodies can fix complement, thereby initiating the complement cascade, or inducing ab-mediated phagocytosis by macrophages. Activated macrophages also release inflammatory and toxic molecules (NO), which predominantly damage ODCs and neurons. Reactive astrocytes induce gliosis at the lesion border. Following inflammatory damage, ODCs proliferate and remyelinate the demyelinated axons.

RA is characterized by destruction of the synovial joints, leading to progressive disability with loss of function. Both genetic and environmental factors are known to contribute to the development of the disease [188]. The reported association between presence of certain HLA D/DR alleles[185,189] and risk of rheumatoid arthritis, together with recognition of MHC class II-expressing antigen presenting cells and T cells in inflamed joints, led to the idea that MHC class II-dependent T cell and B-cell activation were major drivers of the disease [190]. Evidences that T cell homeostasis is not intact in patients with RA, came from the observation that these patients carried large clonally expanded populations of T cells [191,192], comprising 30-50% of synovial tissue cells [193], the majority being $CD4^+$ T cells. Moreover, SKG mice, bearing a point mutation in the ZAP-70 gene, develop autoimmune arthritis, showing a predominant infiltration of $CD4^+$ T cells in the inflamed synovium. Importantly, $CD4^+$ T cells from LNs and spleen can adoptively transfer disease to syngeneic nude or SCID mice, indicating that T cells alone without B cells, can cause chronic arthritis in this strain [194].

Therefore, autoimmune pathogenesis of EAE and SKG may be attributed to self-reactive $CD4^+$ T cells alone, regardless of an additional contribution of other cell types for the pathology. The importance of $CD4^+$ T cells in RA and MS is also highlighted by several observations: self antigen-specific $CD4^+$ T cells and MHC class II antigen-presenting cells are present in inflamed tissues in MS and RA patients[190,195]; the disease is associated to certain MHC II alleles, suggesting that MHC II-restricted $CD4^+$ T cells play a role in pathogenesis[196]; the association between IL-17 present in the serum and synovium of RA patients, and the joint damage [197,198]and $CD4^+$ T cells from healthy donors or patients can respond to self-Ag from the affected tissue in vitro[199]. Overall, $CD4^+$ T cells, even in the absence of $CD8^+$ T cells exhibit autoimmune potential. Moreover, if auto-reactive $CD4^+$ T cells can avoid central tolerance and escape to the periphery, these may be one of the main populations responsible for the development of autoimmunity under certain conditions. Before the discovery of Th17 lineage, pro-inflammatory IFN-γ-producing Th1 cells were thought to play a major role in the pathogenesis of MS, diabetes or RA. Myelin-basic protein (MBP)-specific T cells secreted Th1 type cytokines, and induce disease in naïve mice following adoptive transfer[200]. In addition, mice lacking Th1 associated transcription factors (T-bet and STAT-4) are resistant to the development of EAE[201,202]. Recent evidences point to Th17 subset as the primary contributors, acting as even more potent proinflammatory mediators[55,203]. Autoimmunity still occurs in IFN-γ or IFN-γ-receptor deficient mice, which can be prevented by neutralization of IL-17[204-206]. IL-17 is expressed in the target tissues of patients with numerous autoimmune diseases, and neutralization of this cytokine prevents the development of EAE[207,208]. It is not yet

determined whether the roles of Th1 or th17 cells in autoimmune pathogenesis are mutually exclusive[209,210]. The IL-17 expression is present during acute EAE, while IFN-γ increases and persists for a longer period in the CNS of these mice, suggests that perhaps both subsets cooperate to induce tissue-specific autoimmunity[183].

Overall autoimmunity may arise under certain conditions, depending on the number of self-reactive T cells, the amount and accessibility of self-antigen, the inflammatory cytokine environment, which will favor pathogenic T cells differentiation, and inhibit regulatory T cells expansion, and the functionality and presence of suppressive T cells.

Food Allergy and Anaphylaxis

Food allergy affects approximately 6 to 8% of children less than 3 years old and approximately 2% of the US population[211],having an increasing prevalence worldwide. It is the leading cause of anaphylactic reactions. Severe anaphylaxis is a serious and potentially lethal systemic reaction affecting two or more organs or systems and is due to the release of active mediators from mast cells and basophils.

Normally there is a delicate balance of the gastrointestinal mucosal immune system distinguishing between potentially harmful pathogens, beneficial commensal bacteria, and harmless food allergens which do not induce active immune responses. The mechanisms by which ingested proteins are able to interact with unique populations of APCs leading to cellular and humoral immune responses has been termed oral tolerance.

Loss of oral tolerance can occur or may be bypassed by antigen presentation via alternative routes, such as through cutaneous exposures or via the respiratory tract. Using a murine model, epicutaneous or epidermal exposure to peanut was demonstrated to induce Th2 response and promoted allergic sensitization[212]. In addition, higher rates of peanut allergy have been found in children with atopic dermatitis who used topical creams containing peanut oil[213]. Respiratory exposures are seen in pollen-food syndrome, and IgE-mediated allergy that happens due to cross-reacting proteins in pollens (the initial sensitizing allergen) and foods, which results in oropharyngeal symptoms to raw fruits and vegetables[214].

Oral tolerance breakdown may also occur, as mentioned above, due to defective Tregs function. Atopic dermatitis and food allergies are known manifestations of this disorder[215]. Also, the development of tolerance to milk in children who have outgrown cow's milk allergy, was associated to an increase in circulating Treg levels[216].

Besides, several host and food allergen factors can influence the development of food allergies. Different mouse strains are not equally susceptible to food allergies[217,218], which

suggest the genetic predisposition is important. Murine studies suggest the age of exposure to food allergens can determine whether tolerance or allergy develops[219]. In humans, epidemiologic studies show a higher rate of food allergies in young children as compared to adults [220], but other studies suggest that the early introduction of possible allergens might be beneficial in certain cases[221]. Moreover, other observations show that disruption of normal gut barrier functions, such as gastric pH and commensal bacteria, can increase the risk of food allergies[222]. Mice raised in germ-free environment do not develop normal tolerance[223], and mice treated with antibiotics or those lacking TLR-4 are more easily sensitized to peanut than wild type control mice[224]. C3H/HeJ mice lack functional TLR-4 and do not respond to LPS, but it is not clear how TLR-4 deficiency contributes to the higher susceptibility to peanut allergy in this strain[218]. Furthermore, studies have shown that different patterns of epitope recognition, or epitope diversity, may correlate with clinical manifestations of allergic reactions to peanut and milk. Roasting, which is used to process most peanuts consumed in the United States, increases peanut allergenicity[225]. Additional peanut properties make it a highly allergenic protein. Peanuts contain relatively large quantities of at least 8 proteins that express strong B and T cell epitopes and elicit IgE antibody responses[226,227]. Resistance of peanut allergens to digestion increases the likelihood that more allergens will be absorbed and induce an IgE response and consequently anaphylaxis. Glycosylated Arah1, a major peanut allergen, has been shown to act as a Th2 adjuvant by activating DCs to drive Th2 cell maturation[228]. Recently, peanut proteins were shown to have the ability to induce production of complement (C3a) leading to increased platelet- activating factor and histamine production by macrophages, basophils, and mast cells[229].

Peanut allergy is the most common food-related cause of lethal anaphylaxis, and unlike other food allergies, persists through adulthood[230]. Knowing the different factors involved in triggering an allergic response, several efforts have been made to avoid, and treat allergic disease, but no efficient therapy was found. Nowadays, there is no cure for food-allergy-induced anaphylaxis, but avoidance of the allergen ingestion. C3H/HeJ mice are a suitable model for studying peanut-induced anaphylaxis, as they develop significant levels of specific IgE after sensitization, as well as high plasma histamine levels, and anaphylactic symptoms upon challenge[218].

Having in mind the crucial role for Treg cells establishing immune tolerance, the goal to reestablish dominant tolerance, should rely on Treg cells abilities. This can be achieved by favoring Treg expansion and function, to the degree capable of controlling the effector T cell subsets responsible for the immune imbalance. This way, it may become possible to reset the tolerance state.

1.2.2. Immunomodulation and Tolerance Induction

Allergic and autoimmune disease have one fundamental principle in common: they are triggered by an imbalanced immune system which reacts against a specific challenge, breaking tolerance. Several strategies have been explored to induce peripheral tolerance in experimental models. For instance, tolerance can be induced following treatment with monoclonal antibodies targeting surface molecules on peripheral T cells[231]. Even though, T cell co-stimulation blockade has been successful suppressing immune responses in several studies, it is difficult to suppress responses in animals with memory cells[232]. Concerning depleting therapies, which have already been used in clinical trials[233,234], it is important to bear in mind, that non-specific T cell depletion, may lead to a lymphopenic state promoting extensive T-cell proliferation[235], and consequently, to the generation of new functional memory T cells[236,237].

Nowadays, most current available therapies for autoimmune and allergic diseases are immunosuppressive drugs, which exhibit several associated adverse effects due to the non-specific targeting of the immune response, lifelong administration, and specific toxicity. Among the most serious adverse effects are increased susceptibility to life-threatening infections and cancer. Ideally, the drug should be required for a limited time, and target specifically the cohort of pathogenic cells, leaving the other lymphocytes free to act against infectious agents and malignant cells. For this, it is important to understand how the immune system tolerates self, and use this knowledge to reprogram the system, reinstating its normal balance. Studies in mouse models of human pathology have shown that mAbs targeting key lymphocyte molecules are able to produce long-term tolerance from short-term therapy. This concept became known as immune reprogramming or therapeutic induction of tolerance.

Monoclonal antibodies

Two decades have passed since the initial demonstrations that long-term tolerance can be induced following a brief treatment with mAbs [238-240]. The first studies aimed at interfering with T-cell cooperation, for instance, OKT3, the first mouse antibody specific for human CD3[241], was the first monoclonal antibody used in clinical practice, in the field of transplantation [242,243]. However, the clinical use of OKT3 was hampered by serious side effects linked to its immunogenic and mitogenic potentials[244], which limited its more widespread use in transplantation, as well as its extension to other fields. The mitogenicity of the mAbs led to the design of humanized Abs, engineered to prevent binding to FcRs. In the field of transplantation, some interesting data were published in humans using lymphocyte depletion with alemtuzumab, a humanized anti-CD52 mAb (CAMPATH-1H), as a means of minimizing immunosuppression[245,246]. Some studies demonstrated that mAbs depleting anti-CD4 could enable tolerance to foreign proteins in adult mice [238,239]. Later, tolerance to foreign proteins has been achieved without direct T cell depletion, demonstrating that CD4 co-receptor blockade was sufficient for tolerance induction [163,247]. Since then, many groups have demonstrated that a variety of antibodies that interfere with T cell-APC interactions can also induce immune tolerance. The most efficient have been those ones using co-receptor blockade (CD4 and CD8), and those blocking co-stimulatory molecules, like CD40L (CD154) or CD28 [248-250], this last strategy (with a CTLA4-Ig known as abatacept) was approved for rheumatoid arthritis therapy[251]. In the field of autoimmune disease, a short course of non-mitogenic anti-CD3 antibodies, given at the onset of hyperglycemia, were proven efficient in preventing the progression of type 1 diabetes, improving the clinical manifestations of the disease (namely insulin requirements) for at least two years, which suggests its mechanism being dependent on immune regulation [124,252-254]. This new category of therapeutic agents seems to induce tolerance either through the deletion of alloreactive clones, through the expansion of immunoregulatory T cells, or indeed through both mechanisms[255-257]. In most cases there is some balance between deletion and functional inactivation (anergy) of antigen-specific effector T cells, and conversion or expansion of regulatory T cells, which come to dominate residual antigen-specific T cells.

CD4 co-receptor blockade

Co-receptors such as CD4 contribute to the avidity of the interaction between T cells and antigen presenting cell[258]. Thus, co-receptor blockade or down-modulation is one of the ways of reducing the avidity of T cell/APC interaction and reduce immune responsiveness. In the past decade, the finding that therapeutic tolerance could be

achieved with non-lytic CD4 antibodies, through the expansion of regulatory T cells as major mediators, opened a new field for exploiting mechanisms for drug minimization, with long term effects, in autoimmunity, allergy, transplantation and other immunopathological conditions. Therefore, several attempts were made to use anti-CD4 monoclonal antibodies in pre-clinical studies and even in clinical trials [259-265]. However, inadequate studies and premature assumptions can lead to the abandoning of potentially useful drugs, and this was probably the case for the anti-CD4 [266-269].

Waldmann and coworkers have performed several studies which characterized tolerance induction with anti-CD4 under different conditions, providing important clues concerning the potential usefulness of CD4 blockade. The classical indicator of tolerance is the inability of the host to respond to a later challenge to an otherwise immunogenic form of the tolerated antigen. Mice tolerized to HGG remained indefinitely but specifically unresponsive to subsequent HGG challenges over a long period of time[270]. However, if the subsequent challenge was performed at a late enough time, that the antigen was already cleared from the system, the tolerance could be lost. This led to the assumption that sustained antigen delivery was important for tolerance maintenance. This observation was the very start for studies in transplantation, where the antigen to which the tolerance is induced, is constantly present as part of the transplanted tissue. However, CD4 co-receptor blockade was insufficient to induce tolerance to transplanted tissues, as alloreactive $CD8^+$ T cells would remain free to immediately cause damage to the graft[247]. Therefore, the addition of anti-CD8 to CD4 blockade allowed the induction of tolerance across whole mismatch MHC barriers[271]. Moreover, further studies with the addition of other monoclonal antibodies targeting a co-stimulation receptor – CD154 (CD40L) enabled a more effective control of the remaining activity, allowing the induction of tolerance to fully mismatched allografts, and facilitation of hematopoietic stem cells from fully mismatched donors, in animals that did not receive any drugs or irradiation conditioning, and that possessed an intact immune system [272].

These studies led to a new concept at the time, where tolerance induced by CD4 co-receptor blockade was shown to be *dominant* and *infectious*. Meaning that alloreactive naïve T cells introduced into tolerant hosts, did not reject the graft, but instead, became recruited into the tolerant pool, indicating that the ability to recruit or induce tolerance was itself contagious [86]. This concept was then extended in several other studies [273,274]. These observations were also important in providing an explanation for the long-term maintenance of tolerance in spite of continuous development of new T cells in the thymus.

The discovery of Foxp3 as a major marker for regulatory T cells, allowed to

demonstrate that tolerance induced with CD4 co-receptor blockade could generate *de novo* Foxp3+ regulatory T cells in the periphery. This issue was established in mice devoid of Foxp3+ regulatory T cells by being TCR-transgenic (specific to a male antigen) and RAG-deficient[115]. These Mice could be tolerized to male grafts, and were shown to have CD4+ $CD25^+$ $GITR^+$ T cells expressing Foxp3 in their spleens[115]. Additionally, this tolerization was proved to be critically dependent on TGF-β [115,123]. Mechanistically, anti-CD4 induced transplantation tolerance, is now clearly dependent on the expansion of regulatory T cell, which are found both in the lymphoid tissue and the tolerated transplant[275]. The dominant tolerance state, maintained by Treg cells, allows the resistance to an adoptive transfer of non-tolerant lymphocytes, without tolerance breakdown[270,276]. Additionally these non-tolerant lymphocytes become tolerant and regulatory, when in contact with the first Treg subset – a process called infectious tolerance [86,274,277].

Moreover, studies in our lab have shown that anti-CD4 mAb prevents allergic asthma in murine models, and more importantly induces long term tolerance, as mice remain unresponsive when exposed to the same antigen (Água-Doce, unpublished data). In addition, the antibody-treatment does not compromise the overall immune competence, as treated mice remain able to respond to different antigens.

So far therapies based on CD4 co-receptor blockade seem to be a promising way to induce immune tolerance. Autoimmune diseases are good candidates for such an approach as the antigen is permanently available, which will facilitate tolerance maintenance. My work is based on this principle, of reprogramming the immune system with monoclonal antibodies. The starting point is the hypothesis that even immune pathologies with distinct underlying pathogenesis (namely Th1, Th2, Th17 pathogenesis) could be corrected with a common approach based on the reinforcement of Treg-mediated suppression. Therefore, different animal models of immune deregulation, namely autoimmune and allergic, were used to address that hypothesis.

1.3. Aims of the thesis

Tolerance breakdown is a common problem underlying diseases like autoimmunity and allergy. Autoimmune and allergic diseases constitute a severe burden for people who carry it, suffering a decreasing quality of life, and leading ultimately to death. CD4 T cells are major players in the pathogenesis of these diseases.

For the last half century tolerance induction has been a major goal for immunologists. In the last 20 years, monoclonal antibodies targeting T cell co-receptors or co-stimulatory molecules, have been successfully used in several studies, including transplantation and autoimmunity, being able to induce long term tolerance. Non-depleting anti-CD4 has been extensively studied for the induction of transplantation tolerance. However, its efficacy and mechanism of action in allergic diseases and autoimmunity has not been so thoroughly addressed. This issue is of great importance given the somewhat disappointing results that early clinical trials of anti-CD4 antibodies had in asthma and autoimmune diseases (some of these antibodies were aiming CD4 depletion).

This thesis aims to investigate the ability of non-depleting anti-CD4 monoclonal antibodies to reprogram the immune system in murine models of autoimmune and allergic disease, and identifying the underlying mechanism. In detail it is aimed:

1) To investigate the ability of non-depleting anti-CD4 to prevent the onset and to treat autoimmune responses;
2) To study the efficacy of non-depleting anti-CD4 tolerance induction in a severe allergic response (anaphylaxis).
3) To characterize the mechanisms underlying tolerance induction;

Finally, taken together, the ultimate objective of the thesis relies on the characterization of the potential of non-depleting anti-CD4 monoclonal antibody in tolerance induction, in immune pathology with different types of underlying deregulation (Th1, Th2, and Th17). Such understanding may reveal a need to revisit the clinical application of co-receptor blockade for the treatment of immune pathology.

Autoimmune Arthritis

2. Autoimmune Arthritis

2.1. Background

RA is a chronic systemic inflammatory disease that primarily affects the synovial membranes of multiple joints[188]. A cardinal feature of joint inflammation in RA is proliferative inflammation of synovial cells, i.e., synovitis, which results in the destruction of the adjacent cartilage and bone.

Initial animal models of RA pathogenesis have mainly focused on the role of B cells, since the discovery of one of the main auto-antibodies present in rheumatoid arthritis patients - the Rheumatoid Factor (RF) [278]. Later then, T cells took centre stage[279]. Although $CD4^+$ T cells were assumed to be prime mediators of synovitis, it remained obscure how arthritogenic $CD4^+$ T cells could activate synovial T cells to proliferate, or upon activation, how the autonomous proliferation of synovial cells was maintained, leading to the destruction of the joint. It is now becoming accepted that RA is the consequence of a complex immune deregulation including the contribution of both B and T cells, as well as components from the innate immunity[280].

Both genetic and environmental factors are known to contribute to the pathogenesis of the disease[281]. MHC and non-MHC genes play significant roles in determining the genetic susceptibility to RA[190,281]. Also, various infectious agents, including viruses and bacteria, have been implicated as causative agents of RA[282].

RA is characterized by a complex immune mediated response, integrating multiple aspects of innate and acquired immunity, whose contribution might vary according to disease stage and most probably between patients. Most interactions take place in the synovium, where T cells are activated through the TCR, which recognizes the antigen on the surface of an APC. B cells can function both as antigen presenting cells and antibody producing cells, which deliver antibodies entailed in immune complex formation. Macrophages activated by signals from T cells and by immune complexes produce many proinflammatory cytokines, such as TNF, IL-1, and IL-6, which act synergistically to increase the expression of cell adhesion molecules and cytokine production. The whole inflammatory environment contributes to the production of cartilage-destructive enzymes, and expression of bone-destruction related molecules, such as RANKL [283].

2.1.1. T cells role in autoimmune arthritis

Preliminary evidence that T cell homeostasis is altered in RA patients, came from the observation that these patients carried large clonally expanded populations of T cells [191,192], comprising 30-50% of synovial tissue cells [193]. The majority are $CD4^+$ T cells, although $CD8^+$ T cells are also present and might also contribute to the pathogenesis [284]. Depending on the cytokine environment, CD4 T cells might show different phenotypes. Th1 type of response has been for a long time associated to autoimmune diseases, such as diabetes, multiple sclerosis and RA. Recently, a new subset of $CD4^+$ T cells, named Th17, characterized by IL-17 production , were described to be involved in RA as well as in other autoimmune diseases[285]. Furthermore, several studies have reported an association between IL-17 levels, in the serum and synovia of RA patients, and joint damage [197,198]. IL-17 is a highly pleiotropic cytokine with effects on a variety of cell types, such as monocytes, fibroblast-like synoviocytes, chondrocytes and osteoclasts. These effects include inflammation, angiogenesis, osteoclastogenesis, and breakdown of bone and cartilage. So far, the distinct contribution of Th1 and Th17 to the pathogenesis of the disease is still unclear [209,210].

Importantly, one should bear in mind that pathogenic T cells may not always resemble conventional, antigen-activated T cells [286]. In RA patients lymphocytes show phenotypic and functional signs of premature immunosenescence. Clonally expanded $CD4^+$ T cells were shown to lose CD28 expression, indicating a shift in their functional profile, and were consistently auto-reactive [192]. Accumulation of senescent $CD4^+$ T cells correlates with a defect in producing new T cells from the thymus, suggesting premature failure of thymopoiesis as the underlying mechanism. Importantly, regulatory T cells, which are known to have an important role in autoimmunity prevention, have been shown to be defective in several RA studies[287]. In juvenile rheumatoid arthritis, disease activity was found to correlate with defective numbers and/or activities of Tregs[288]. TNF is abundant in the sera of RA patients, and it was shown to inhibit suppressive function of Tregs, which was restored by anti-TNF therapy[287,289]. Accordingly, regulatory T cells from RA patients were shown to be less able to suppress the production of IFN-γ and TNF by $CD4^+$ T cells, even though they can suppress their proliferation[287,289]. Moreover, Treg cells from patients with active RA show reduced expression of Foxp3 (which is known to be crucial for their proper function). IL-6, which has been shown to be abundantly present in the rheumatoid synovium, and is associated to Th17 differentiation, has also been shown to

render T effector cells resistant to Treg-mediated suppression[290]. Most human studies, focused on Tregs from the peripheral blood or joints, and it is not clear whether Tregs from different anatomic compartments are functionally equivalent, as they are exposed to different cytokine environments. It is still not yet established whether synovial Treg cells are in fact active *in vivo*, and if so, which are the cells they target (synovial fibroblasts, macrophages, mast cells, neutrophils, etc), and whether or not they exert their immunomodulatory function in an antigen-specific, cytokine-dependent, or contact-dependent manner. To clarify these issues, several animal models have been used. Importantly there has been an increasing effort to find better models which closely reproduce human disease.

2.1.2. Animal Models

Much of our current understanding of autoimmune arthritis has been based on collagen-induced arthritis (CIA) in mice, where type II collagen (CII) immunization and challenge induce a robust T-cell dependent autoantibody response that promotes joint inflammation. In addition to antigen-induced arthritis like CIA or adjuvant arthritis, recent efforts with transgenic, gene-knockout, or gene-knock-in technology have established several new animal models of autoimmune arthritis (see table 1), for instance through overproduction of proinflammatory cytokines[291-293]. Some animals develop arthritis through expression of transgenic TCR, like in the case of K/BxN mice (offspring of KRN T cell receptor transgenic mice crossed with NOD mice), who bear a transgenic TCR which recognizes a peptide of glucose-6-phosphoisomerase (G6PI), and the mice spontaneously produce antibodies anti-G6PI, which is highly arthritogenic [294].

SKG mice which have a point mutation of the gene encoding the carboxy-terminal SH2 domain of ZAP-70, a key signal transduction molecule in T cells, and shows an autossomal recessive inheritance of the disease were also shown to develop spontaneous arthritis, with several clinical and immunological features resembling human RA[194].

Induction	Animal model	Species	Description, main players	Ref
Non-specific stimuli	Adjuvant induced arthritis – **AA**	Lewis rat	Autoimmune, T cell mediated	295
	Oil-induced arthritis- **OIA**	DA rat	Autoimmune, T cell mediated	296
	Pristane-induced arthritis	DA rat	Autoimmune, T cell mediated	297
Cartilage directed	Collagen-induced arthritis - **CIA**	DBA mouse	Anti-collagen II autoimmune, immune complexes and T cells	298
	Proteoglycan-induced arthritis - **PGIA**	Balb/c mouse	Anti-proteoglycan autoimmune, immune complexes and T cells	299
Exogenous/ Infectious agents	Steprtococcal cell wall arthritis – **SCW-A**	Lewis rat	T cell mediated	300
	Antigen-induced arthritis **- AIA**	Mouse	Th17 mediated	301
Transgenic spontaneous	**SKG** arthritis	SKG	ZAP-10 mutation, chronic disease, T cells mediated (Th17), RF production	194
	KRN arthritis	K/BxN (KRNxNOD)	Transgenic TCR against G6PI, G6PI autoimmunity, Aabs and T cells	302
	TNF transgenic arthritis	TNFtg mouse	TNF overexpression	292
	IL-1 transgenic arthritis	IL-1tg mouse	IL-1 overexpression	293
	HTLV-induced arthritis	Mouse	transgenic expression of human T-lymphotropic virus-1 (HTLV-1) tax protein, T cells	303
	MRL-lpr/lpr mice		mutation in the Fas gene chronic arthrtitis, production of RF	304
Immune complexes	Collagen type II - **CAIA**	DBA mouse	Mouse CII antibodies	305
	KRN serum	Balb/c mouse	Mouse GPI antibodies	306

Table 1 – Animal models of autoimmune arthritis – This table represents some of the most commonly used models of autoimmune arthritis, in rodent species

SKG mice

SKG mice develop an autoimmune arthritis resembling human RA in clinical, histological and serological features. Clinically, hyperemia of finger joints becomes evident around 2 months of age, progressing in a symmetrical fashion to swelling of the finger joints, of the fore and hind paws, and subsequently larger joints (wrists and ankles). The course of the disease is chronic, and more severe in female than male animals. Histologically, SKG arthritis show severe synovitis with massive subsynovial infiltration of neutrophils, lymphocytes, macrophages and plasma cells, villus proliferation of synoviocytes accompanying pannus formation and neovascularization. With progression

of synoviocyte proliferation, pannus erodes adjacent cartilage and subchondral bone (reviewed in[307]).

Serologically SKG mice develop high titers of RF and auto-antibodies specific for CII, which is highly specific for human RA. $CD4^+$ T cells predominantly infiltrate the subsynovial tissue and mediated chronic autoimmune arthritis. $CD4^+$ T cells in the spleen and LNs can adoptively transfer the disease to syngeneic nude or SCID mice, indicating that T cells alone without B cells, can cause chronic arthritis in this strain [194]. Furthermore, thymocyte transfer from arthritic or non-arthritic SKG mice can also induce disease in SCID mice, indicating the thymic production of arthritogenic T cells [194].

Proinflammatory cytokines such as TNF, IL-1 and IL-6 are abundantly produced in the affected joints of SKG mice[308], and the severity of the disease is significantly reduced when the mice are rendered, TNF, IL-1 or IL-6-deficient. Moreover, since Th17 were described, it was shown that the development of arthritis in these mice is Th17-dependent, as IL-17 KO mice do not develop disease [309]. In fact, self-reactive T cells produced in SKG mice as a result of genetically altered thymic T cell selection spontaneously differentiate into IL-17-secreting $CD4^+$ T cells, driven by APC-derived IL-6 together with T cell-derived IL-6[309]. Recently, Hashimoto et al reported that this IL-6 production is triggered by the C5a component of complement activation, which is activated by β-glucan (one of the components that provokes autoimmune arthritis in SKG mice), and consequently leads to the stimulation of tissue-resident macrophages that produce inflammatory cytokines such as IL-6 in synergy with GM-CSF. Moreover, C5a deficiency and macrophage deficiency prevents arthritis development in these mice[310].

ZAP-70 mutation

There is accumulating evidence that genetically determined anomaly or variation in T cell signaling predisposes the host to autoimmunity. Autoimmune arthritis develops in SKG mice as a consequence of a general abnormality of the T-cell signal transduction through ZAP-70. These mice bear a point mutation in the carboxyl-terminal Src homology 2 domain of ZAP-70, a highly T-cell restricted signaling molecule[194]. This mutation and altered signal transduction in T cells, affects thymic development and differentiation of T cells, in particular their positive and negative selection, responsiveness of mature cells to self- and non-self-antigens, and the generation and function of $CD4^+CD25^+Foxp3^+$ natural regulatory T cells[311]. In humans, Zap-70 deficiency affects thymic positive selection of CD8+ T cells and the function of $CD4^+$ T cells[312,313]. In the case of SKG mice, these alterations in thymic T cell selection result in the skewing of the whole T cell repertoire

toward higher self-reactivity, including arthritogenic clones, hampering positive selection of weakly self-reactive T cells. The degree of this selection shift will depend on the homo or heterozygosity of the *skg* mutation[314]. Importantly, the ZAP-70 mutation affects simultaneously the repertoire of Foxp3$^-$ conventional T cells as well as Foxp3$^+$ natural regulatory T cells, skewing the latter to a higher self-reactivity than the former[314]. Moreover, as probably expected, suppressive function of nTregs was also affected, since Tregs require TCR signaling to exert their function. However, the pro-inflammatory environment, in the presence of IL-6 production, may also render T effector cells resistant to Tregs suppression, and inhibit the generation of peripherally induced Foxp3$^+$ T cells, thus facilitating arthritis progression[55,57].

2.1.3. Therapeutic strategies

Strategies for treatment of rheumatoid arthritis have changed greatly over the past decade, in an attempt to reduce inflammation (and as a consequence, joint damage), and to target specific molecules known to be involved in the pathogenesis of the disease. T cells are known to have an important role in the pathogenesis of the diseases and therefore, have been considered legitimate targets.

For a long time disease-modifying anti-rheumatic drugs (DMARDs) were the only available treatment, and were highly beneficial for control of inflammatory activity and development of erosions in many patients[315-317]. A number of conventional DMARDs, besides being potent immunosuppressive drugs, interfered with T-cell function. Namely, cyclosporine, leflunomide and methotrexate (MTX) inhibit certain pathways affecting T cell proliferation and activation [318-320].

The discovery of monoclonal antibodies in the 70s allowed to target specifically the main molecular mediators involved in the pathogenesis of RA. The first biological therapies applied experimentally to RA patients targeted T cells. These ranged from lymphocytotoxic-depleting therapies, such as CAMPATH-1G and CAMPATH-1H (alemtuzumab) to depleting and non-depleting CD4 monoclonal antibodies. Depleting anti-T cell monoclonal antibodies ended up being disappointing, once it targeted all T cells indiscriminately, causing several adverse effects, namely prolonged lymphopenia after treatment. Furthermore, depleting antibodies are more efficient eliminating naïve T cells, than activated effector cells. Therefore, anti-T cell depleting Mabs have been abandoned in favor of strategies aiming to induce tolerance within the T cells. The administration of non-depleting anti-CD4 antibody before the appearance of arthritis in CIA model prevented disease development, reducing IFN-γ and increasing IL-4 levels, suggesting a

protective Th2 shift in the immune response[321]. Even though both depleting[322-324] and non-depleting[259,260,265,269,325] anti-CD4 monoclonal antibodies have been shown to be effective in the treatment of autoimmunity in mouse models, their mechanism of action was different. The encouraging pre-clinical data with non-depleting anti-CD4 led to their use in some clinical trials[269,326,327]. However, several patients developed adverse effects, such as skin rashes, and the treatment was discontinued[328]. It is likely that these potentially tolerogenic drugs were prematurely and inappropriately abandoned, without considering doses, time points, epitope targeting, and a series of factors that are known to influence their efficacy *in vivo*. It should be noted that these trials took place at a time little was known about the therapeutic use of monoclonals, and the molecules tested were not humanized and highly immunogenic. A humanized anti-CD4 mAb was recently published to be effective in synergy with anti-TNF, and successfully causing no atypical infections[329].In addition, the effect of three distinct anti-CD4 antibodies (targeting different epitopes) in CIA led to different patterns of disease development[330].

The first key contribution in the development of treatments targeting specific molecules of the immune system, was made by scientists at the Kennedy Institute in London, who characterized TNF as having an important role in the pathogenesis of rheumatoid arthritis, doing a small clinical study of TNF blockade in patients with this disease[331], and later confirmed their findings with randomized clinical trials[332,333]. Nowadays, there are several TNF-blocking agents approved for clinical use. TNF blockade is usually more effective when combined with MTX [334]. TNF-blockade efficacy may lie on the manipulation of the pro-inflammatory environment, favoring the suppressive function of Tregs. This is supported by several observations in RA patients, where chimeric monoclonal anti-TNF infliximab, resulted in an increased number of Foxp3+ T cells in responding patients[287,321].

The success of TNF-blockade, rapidly led to the development and testing of a series of biological drugs, targeting several other molecules, in different inflammatory pathways. First IL-1 binding to its receptor was inhibited, but its effectiveness was not as good as TNF-blockade[335]. Then, a mAb against IL-6 receptor was tested and approved for clinical use [336]. Rituximab, a monoclonal antibody targeting CD20 on B lymphocytes was also shown to be effective in RA, and it is, nowadays, licensed for clinical use[337]. Moreover, co-stimulatory blockade came up as a promising tool, not only for preventing T cell activation, but for tolerance induction[338]. CD28 targeting with a CTLA4-Ig fusion protein (abatacept) was successful in clinical trials and it is now licensed for RA treatment, being the only drug blocking co-stimulation accepted for clinical use[339].

CTLA-4 is a negative regulator of T cells that is up-regulated after T cell activation, and

has a higher affinity to CD80/86 than CD28. Therefore CTLA4-Ig interrupts the co-stimulatory signal, preventing and potentially reversing T-cell activation. The Fc modification prevents complement activation, reducing the incidence of infusion reactions[340]. In fact, patients who do not meet treatment goals on their first round for TNF blockade, might change strategy and start using abatacept or rituximab. Even though new insights have been used to develop new and very efficient treatment approaches for patients, still the efficacy of each treatment differs from patient to patient, and with the time of treatment. Ideally, if the immune system could be targeted at the very beginning of the burst of the pathogenic response and change permanently its destructive behavior, we would be in the right path. As $CD4^+$ T cells are the main triggers for an autoimmune response, with the enhanced understanding surrounding immune regulation, and T helper subsets, we decided to re-evaluate the mechanisms underlying tolerance induction with non-depleting anti-CD4.

2.2. Material and Methods

Mice. BALB/c, DO11.10.RAG1$^{-/-}$, and SKG mice (generously provided by Professor Shimon Sakaguchi, Kyoto, Japan) were bred and maintained under specific pathogen free (SPF) conditions at the Instituto Gulbenkian de Ciência. Experimental animals were between 8-10 weeks of age and sex matched. All experiments involving animals were conducted according to guidelines from the Animal User and Institutional Ethical Committees.

Autoimmune arthritis induction and anti-CD4 treatment. BALB/c and SKG mice were injected intraperitoneally (i.p.) with a single shot of 3 mg curdlan per mouse. Treated mice were injected with 1 mg anti-CD4 on days 0 (the day of curdlan injection), 2 and 4. Mice treated at disease onset, were injected with 3 shots of 1 mg anti-CD4 every other day, from the day they have reached a clinical score of 0.5.

Clinical assessment of arthritis. Joint swelling was monitored in blinded cages by two independent observers and scored as described elsewhere [194]: 0, no joint swelling; 0.1, swelling of one finger joint; 0.5, mild swelling of wrist, ankle, or base of tail; and 1.0, severe swelling of wrist, ankle or base of tail. Scores for all joints were totaled for each

mouse (with a maximum score of 5 corresponding to severe swelling of the four paws and base of tail).

Antibodies and reagents. Curdlan (Wako, Japan) and Zymosan-A (Sigma-Aldrich, USA) were dissolved in sterile PBS at 15 mg/ml and 20 mg/ml, respectively. Non-depleting anti-CD4 (YTS177) and the isotype control anti-dog CD4 (YKIX302) Mabs were produced in our laboratory using Integra CL1000 flasks (IBS, Chur, Switzerland), purified by 50% ammonium sulfate precipitation, dialyzed against PBS, and purity checked by native and SDS gel electrophoresis. The hybridomas were generously provided by Professor Herman Waldmann (Oxford, UK).

Cytokine Analysis. Evaluation of serum cytokines was performed using the mouse inflammation cytometric bead array (BD Biosciences, San Diego, CA), with beads specific for IL-6, TNF, IFN-γ, monocyte chemoattractant protein-1 (MCP-1), and IL-10. IL-17 was quantified by ELISA using a kit from R&D Systems. Cytokine concentrations were measured in duplicates, and compared with standard curves, according to manufacturer instructions.

Determination of ovalbumin-specific immune responses. On day 30 following anti-CD4 treatment, or treatment with an isotype control, the mice were immunized with two injections two weeks apart, of 20 µg ovalbumin (OVA, grade V; Sigma, St Louis, USA), in 2.0 mg of endotoxin-free aluminum hydroxide (alum, Alu-gel-S, Serva, Heidelberg, Germany), and sacrificed one week after the last immunization. Non-immunized (naïve) mice were maintained as negative controls. The serum concentration of OVA-specific immunoglobulins was determined by ELISA using an OVA-specific IgG1 kit (SouthernBiotech, Birmingham, USA) with anti-OVA IgG1 standard from Serotec (Oxford, UK).

Histology. Ankle and metatarso-phalangical (MTP) joints were collected and cryopreserved in OCT (Sakura, NL). Cryosections were stained with hematoxilin-eosin according to standard procedures.

Flow Cytometry. Cells were stained for flow cytometric analysis with the following fluorochrome-labeled monoclonal antibodies: CD3 (145-2C11), CD4 (RM4-5), CD25 (PC61.5), and Foxp3 (FJK-16s) from eBiosciences or BD Biosciences. Intracellular cytokines were investigated in lymphocytes activated for three hours in complete RPMI, containing phorbol 12-myristate 13-acetate (PMA, 50 ng/ml), ionomycin (500 ng/ml) and Brefeldin-A (10 mg/ml), at 37º C. Monoclonal antibodies specific for IFN-γ (XMG1.2), IL-17A (eBio17B7), and IL-10 (JES5-16E3) were used (all from eBiosciences).

Th17-polarization assays. OVA-specific CD4+ T cells from DO11.10.RAG1-/- mice were purified by magnetic separation with CD4 (L3T4) microbeads (Miltenyi Biotec, Germany). Cell purities were between 92-96%. The T cells were cultured for 5 days with bone marrow derived DCs, 0.1 µM OVA peptide (New England Peptide LLC, USA), and 10 µg/ml anti-CD4 (YTS177) in IMDM 5% FBS (Invitrogen), 1% Pen/Strep (GibCo), 0.1% β-Mercaptoethanol (GibCo), 1 ng/ml TGF-β (R&D systems), 20 ng/ml IL-6 (R&D systems), 10 ng/ml IL-1β (Ebiosciences), and 10 µg/ml anti-INF-γ (R46A2). At the end of the culture the cells were harvested, and processed for flow cytometry.

RNA extraction and real time PCR. Total RNA was extracted from synovial tissue, dissected from ankle joints, using lysis buffer, and following the tissue RNA kit instructions (Omega bio-tek, USA). Foxp3 and IL-17 were quantified by real time PCR, performed on the ABI Prism® 7000 sequence detection system (Applied Biosystems, USA). The relative mRNA levels of the target genes were normalized against CD3. CD3, Foxp3 and IL-17 primers are described elsewhere [65,115,341].

Statistics. Statistical significance was determined using the two-tailed non-parametric Student's t test (Mann-Whitney U). *P* values <0.05 were deemed significant.

2.3. Results

2.3.1. SKG mice develop chronic autoimmune arthritis upon systemic curdlan immunization

Although initial reports have suggested that SKG mice develop chronic autoimmune arthritis spontaneously[194], it was later confirmed that disease induction requires exposure to yeast wall extract (zymosan) or purified β-glucans, like curdlan or laminarin, acting through the pattern recognition receptor Dectin-1[342].

We confirmed that a single i.p. injection of 2 mg Zymosan (not shown) or 3 mg Curdlan is sufficient to induce chronic disease in SKG mice, but not in wild-type controls (Figure 1A). Both male and female mice developed arthritis, although the disease was more severe in females (Figure 1B). Based on these data we decided to use curdlan in female SKG mice in subsequent experiments.

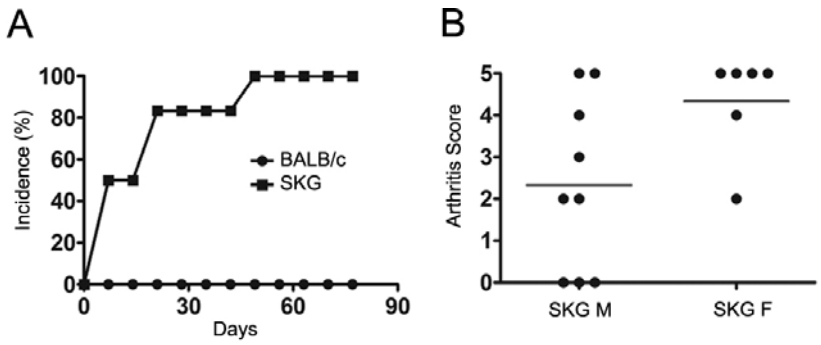


Figure 1 - SKG mice develop chronic autoimmune arthritis upon induction with curdlan. **(A)** Arthritis incidence in female SKG and BALB/c mice after a single i.p. injection of 3 mg curdlan (n=6). Curdlan was able to induce chronic arthritis in SKG but not in BALB/c mice. The graph represents the median per group. **(B)** Clinical score of 5 month-old male (M) and female (F) SKG mice 90 days following disease induction with curdlan. Data are from two independent experiment.

2.3.2. Non-depleting anti-CD4 treatment prevents the onset of autoimmune arthritis

To assess whether non-depleting anti-CD4 Mabs, suggested in previous studies as having tolerogenic potential, can lead to long-term beneficial effects in chronic autoimmune arthritis, female SKG mice were treated with non-depleting anti-CD4 together with curdlan. Anti-CD4 treatment was effective in preventing the development of autoimmune arthritis (n=5, $P<0.001$, Figure 2A). Rheumatoid factor, an immune complex which commonly characterizes SKG autoimmune arthritis and human RA, was not detectable in anti-CD4 treated mice (Figure 2B).

Furthermore, animals treated with anti-CD4 not only remained without clinical manifestations of the disease, but their joints were free from inflammatory cell infiltrates. Indeed, histological sections of ankle and MTP joints from anti-CD4 treated mice showed normal joint tissues, without inflammatory infiltration or bone erosions, compared to the curdlan induced arthritic mice (Figure 2C).

We also evaluated the presence of pro-inflammatory cytokines in arthritic mice compared to anti-CD4 treated animals. We observed a significant decrease in the concentration of IL-6, TNF, MCP-1 and IFN-γ in sera from anti-CD4 treated mice, suggesting that a treatment targeting T cells can have an impact on additional cell types producing those cytokines (Figure 2D). It should be noted that IL-6, which has been associated with Th17 differentiation, is known to be critical for the pathogenesis of chronic autoimmune arthritis in SKG mice [343], and was not detectable in any of the anti-CD4 treated animals. Despite the reported association of IL-17 with arthritis in SKG mice, this cytokine was not detected in sera from arthritic animals, remaining below detection level in all groups (Figure 2E). This observation is in accordance with the proposed local effect of this inflammatory mediator.

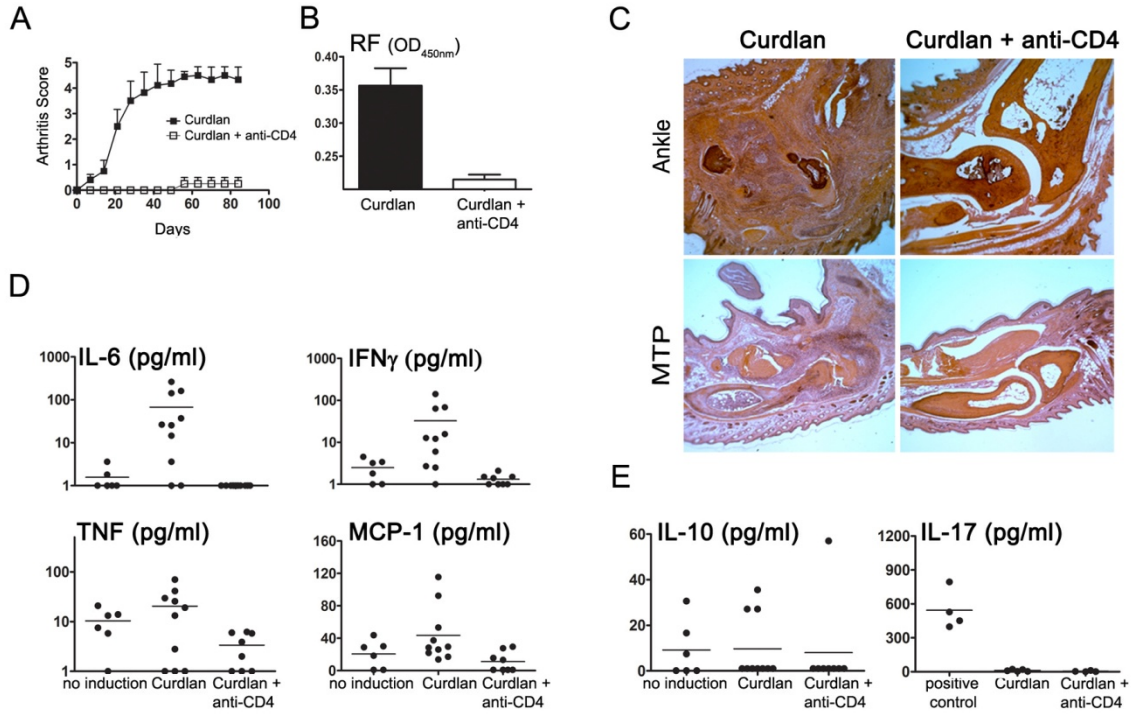


Figure 2. Non-depleting anti-CD4 MAb prevents the onset of autoimmune arthritis. - (A) Female SKG mice were immunized with 3mg curdlan i.p. together with 1 mg non-depleting anti-CD4 or an isotype control. The MAb was administered again on days 2 and 4. Anti-CD4 treated mice were protected from the development of autoimmune arthritis (n=6, ****P<0.001*). Data, represented as mean ± SEM, are from two independent experiments. **(B)** Serum concentration of rheumatoid factor (RF) was measured by ELISA. Mice treated with anti-CD4 showed significantly lower levels of RF (n=6, ****P<0.001*). **(C)** Histological sections stained with eosin-hematoxilin from the ankle and metatarso-phalangical joints from SKG mice in the absence and in the presence of anti-CD4 treatment, 90 days following curdlan immunization. **(D)** Serum concentration of IL-6, IFN-γ, TNF and MCP-1 in naive SKG mice, SKG mice exposed to curdlan, or curlan and anti-CD4. Naive SKG mice were age matched and did not develop arthritis in the absence of curdlan immunization (no induction). The serum levels of IL-6 (**P<0.05*), IFN-γ (***P<0.01*) and MCP-1 (***P<0.01*) were significantly lower in anti-CD4 treated mice compared with animals injected with curldan in the absence of tolerizing MAbs. Differences in TNF concentration did not reach statistical significance. **(E)** The serum concentration of IL-10 and IL-17 in SKG mice exposed to curdlan, or curlan and anti-CD4 remained similar in the different experimental groups. Culture supernatants from Th17 cell culture were used as positive control.

Previous studies have suggested a protective role for IL-10 in this murine model of autoimmune arthritis [308]. However, we did not find any significant difference in the serum levels of IL-10, between arthritic and anti-CD4 treated mice (n= 10, Figure 2E).

2.3.3. Non-depleting anti-CD4 alters the balance of Treg/Th17 cells in the synovial tissue and draining LNs

It has been reported that the anti-CD4 MAb used in our study (YTS177) has a non-depleting isotype [247]. We confirmed the non-depleting nature of the MAb as neither the splenic frequency (Figure 3A and B) nor the total number (not shown) of $CD4^+$ T cells were reduced in animals treated with anti-CD4.

Transplantation tolerance achieved with non-depleting anti-CD4 has been associated with the induction of Treg cells, both in the spleen and within tolerated transplants [275,344,345]. We investigated if changes in Treg frequency could be seen in the spleen of anti-CD4 treated mice. We found that there was no change in T cell subpopulations of anti-CD4-treated animals, namely $Foxp3^+$ Treg cells, with a similar frequency of these cells in mice exposed to curdlan in the presence or absence of anti-CD4 treatment (Figure 3A and B). In addition, the frequency of IL-17, IFN-γ or IL-10-producing T cells, identified by intracellular cytokine staining, remained constant in all groups of animals, and below 2% of the $CD4^+$ T cells (not shown).

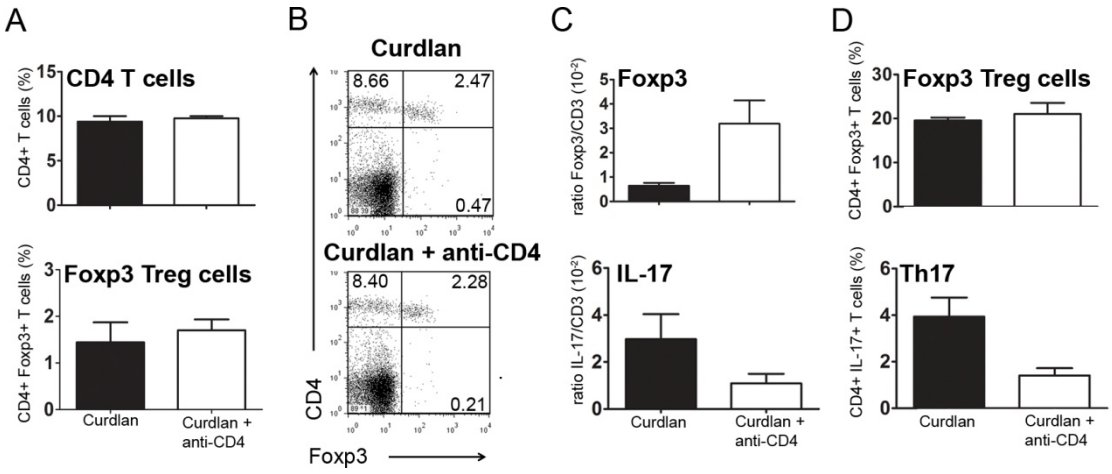


Figure 3. Anti-CD4 MAb influences the local balance of Th17/Treg cells. - **(A)** Frequency of splenic CD4+ T cells or CD4+Foxp3+ Treg cells from SKG mice exposed to curdlan, or curdlan + anti-CD4 treatment. No significant difference was observed between the two populations of animals. **(B)** Representative dot plots showing the frequency of splenic CD4+Foxp3+ T cells from SKG mice exposed to curdlan, or curdlan + anti-CD4 treatment. No significant difference was observed, as represented in panel A. **(C)** Foxp3 and IL-17 mRNA expression from the synovial membrane of SKG mice exposed to curdlan, or curdlan + anti-CD4 (mRNA expression levels were normalized to CD3 expression). **(D)** Frequency of Foxp3+ and IL-17+ T cells within draining LNs of SKG mice exposed to curdlan, or curdlan + anti-CD4.

As our data show that anti-CD4 treatment can prevent joint inflammation, even though T cell subpopulations in secondary lymphoid organs appear to be unaffected, we investigated whether anti-CD4 treatment was leading to alterations in the T cell subpopulations within the synovial tissue of protected animals. Given the technical limitations for the direct enumeration of individual T cells from mouse synovial tissue, we used the expression of Foxp3 and IL-17 mRNA as surrogate markers for the relative frequency of, respectively, Tregs and Th17 cells. For this purpose Foxp3 and IL-17 mRNA expression was measured by quantitative real time PCR and normalized to CD3 expression (thus controlling for different numbers of infiltrating T cells in different samples, as the arthritic synovium contains greater numbers of T cells). This method, of using CD3 expression for normalization of different numbers of infiltrating T cells in tissues with few lymphocytes has been established for other tissues with small numbers of T cells, such as skin grafts in mice [115]. We observed that while IL-17 expression among synovial T cells (i.e. IL-17/CD3 mRNA ratio) was reduced in mice treated with anti-CD4 and consequently protected from inflammatory manifestations of the disease, the expression of Foxp3 among synovial T cells was increased in the same conditions (n=4, $P<0.05$, Figure 3C). Thus, the Foxp3/Th17 ratio in the synovial tissue is substantially shifted following anti-CD4 treatment. In addition, the analysis of popliteal LNs, draining affected joints, showed a reduction in the frequency of IL-17$^+$ T cells in anti-CD4-treated mice (n=8, $P<0.05$) while the frequency of Foxp3$^+$ cells remained similar in both groups of animals (Figure 3D).

2.3.4. CD4-blockade prevents in-vitro Th17 polarization while favoring Foxp3 expression

Although non-depleting anti-CD4 has been widely studied for the induction of transplantation tolerance its exact mechanism of action has not been fully characterized. In experiments performed with TCR-transgenic mice devoid of Foxp3$^+$ Treg cells, it was previously shown that CD4-blockade could directly lead to peripheral conversion of T cells towards Foxp3$^+$ Treg cells thus achieving transplantation tolerance [115]. However, it was never assessed whether CD4-blockade could directly interfere with Th17 polarization, or whether a reduction in Th17 cells (as we observed *in vivo*) would be secondary to Treg-mediated suppression.

To address this issue we investigated whether CD4-blockade could prevent *in vitro* Th17 polarization. We used OVA-specific TCR-transgenic CD4$^+$ T cells sorted from DO11.10 RAG$^{-/-}$ mice. We stimulated these cells *in vitro*, for five days, in the presence of DCs loaded

with the appropriate peptide and under culture conditions promoting optimal Th17 polarization (in presence of TGF-β, IL-6, IL-1β, and anti-IFN-γ)[57]. We observed that addition of anti-CD4 led to a significant reduction of Th17 cells (n=6, *P*<0.05, Figure 4). In spite of the presence of cytokines that promote Th17 polarization (cytokines that are known to prevent Foxp3 induction) the addition of anti-CD4 resulted in a significant increase in the frequency of Foxp3$^+$ T cells (n=6, *P*<0.05, Figure 4).

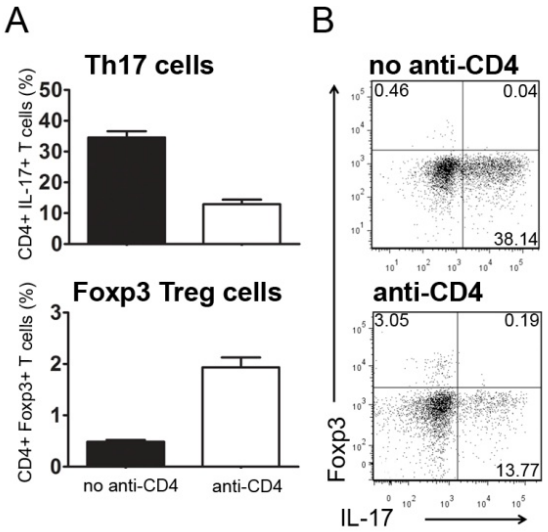


Figure 4. CD4-blockade prevents Th17 polarization. - **(A)** Sorted TCR-transgenic cells were stimulated in vitro with 0.1 μM peptide-loaded DCs under culture conditions known to preferentially polarize Th17 cells, with the addition of recombinant TGF-β, IL-6, IL-1β, and anti-IFN-γ. After 5 days of culture we observed a significant reduction of IL-17+ cells in the presence of 10 μg/ml anti-CD4 (n=6, **P<0.05*). In contrast, anti-CD4 addition led to an increased frequency of Foxp3+ T cells (n=6, **P<0.05*). **(B)** Representative dot plots from the two different culture conditions.

2.3.5. Non-depleting anti-CD4 induces long- term protection from autoimmune arthritis

To assess whether protection from arthritis induced with anti-CD4 treatment had a long-term effect, SKG mice initially injected with curdlan and non-depleting anti-CD4 were challenged with curdlan 60 days following the initial treatment. Animals exposed to curdlan in the presence of the putative tolerogenic anti-CD4 MAb at day 0 were protected from the induction of arthritis following curdlan administration at day 60, unlike the age-matched controls that did not receive any treatment at day 0 (n=6, *P*<0.05, Figure 5A).

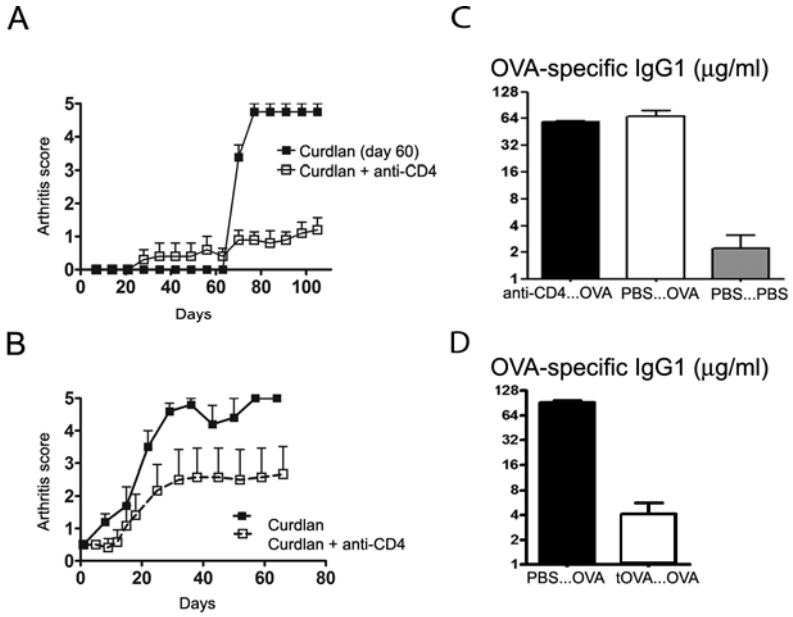


Figure 5 - Long-term protective effect of non-depleting anti-CD4 treatment does not affect immune competence. - (A) Female SKG mice were injected with 3 mg curdlan i.p. on day 0 and anti-CD4 on days 0, 2, and 4. Animals treated with anti-CD4 displayed long term protection from arthritis (n=5, *$P<0.05$). On day 60, anti-CD4 treated mice and age matched SKG were challenged with 3 mg curdlan i.p. Mice previously treated with anti-CD4 remained protected from the development of autoimmune arthritis (n=5, *$P<0.05$). **(B)** Female SKG mice were injected with 3 mg curdlan i.p. and, when the clinical score reached 0.5, some of the animals initiated treatment with three i.p. administrations of 1 mg anti-CD4 on alternate days. Control animals rapidly progressed to severe arthritis unlike the anti-CD4 treated mice (n=6, *$P<0.05$). **(C)** Mice were treated with non-depleting anti-CD4 30 days before immunization with OVA-alum i.p. (αCD4…OVA). One week following sensitization the serum levels of OVA-specific IgG1 were quantified, and compared with untreated (PBS…OVA) and non-immunized controls (PBS…PBS). Mice treated with anti-CD4 MAbs were competent to produce OVA-specific IgG1 to titres similar to untreated controls, and considerably higher than the non-immunized mice (n=5, *$P<0.05$). **(D)** OVA-specific IgG1 in mice where OVA was initially administered at the time of anti-CD4 treatment (tOVA…OVA), compared with animals that were not initially treated with anti-CD4 (PBS…OVA). Mice treated with anti-CD4 at the time of sensitization with OVA, became tolerant to the subsequent OVA immunization (n=6, *$P<0.05$).

However, it should be noted, that some anti-CD4 treated mice developed mild manifestations of arthritis following curdlan challenge at day 60, although without progressing to the severe manifestations of the disease observed in control groups.

2.3.6. Non-depleting anti-CD4 prevents progression of established autoimmune arthritis

Given the fact that non-depleting anti-CD4 treatment prevents the onset of autoimmune arthritis, we tested the effectiveness of a similar course of anti-CD4 for the treatment of established arthritis in SKG mice. Female SKG mice were immunized with curdlan and when the clinical score reached 0.5 the arthritic mice were randomly included in a group treated with anti-CD4 Mab or a control group. Mice treated with non-depleting anti-CD4 showed a long-term benefit with slower disease progression and less severe clinical scores (n=5, $P<0.05$, Figure 5B). However, remission was only achieved in a minority of the treated animals.

2.3.7. Anti-CD4 treated mice remain immunocompetent

A concern of immunomodulatory or tolerogenic therapeutic strategies is their long-term impact on the overall immune response. We therefore assessed the immune competence of anti-CD4-treated BALB/c mice (same genetic background as the SKG mice, but without the ZAP-70 point mutation) to mount $CD4^+$ T cell-dependent immune responses towards an unrelated antigen. For this purpose, mice treated with anti-CD4 were sensitized with 20 µg OVA-alum 30 days following anti-CD4 treatment. The quantification of OVA-specific immunoglobulins in the serum was determined one week following sensitization. Our data show that the concentration of OVA-specific Igs was similar in immunized mice, regardless of previous anti-CD4 treatment, and considerable higher than in naive controls (n=5, $P<0.05$, Figure 5C). Moreover, we confirmed the same results in SKG mice, subjected to the same protocol (n=2, not shown). Of note, if OVA was administered at the time of anti-CD4 treatment in BALB/c mice, the animals became unable to produce OVA-specific Igs following subsequent OVA immunization (n=6, $P<0,05$, Figure 5D), thus proving that OVA-specific IgG production is CD4-dependent, and that tolerance is only imposed over the antigens present at the time of tolerance induction.

2.4. Discussion

Our data show that a short course of non-depleting anti-CD4 can lead to long-term protection from the development of autoimmune arthritis, in a murine model of chronic disease. We have shown that our non-depleting antibody (clone YTS177) is not affecting T cells in the spleen, but seems to be preventing autoimmune arthritis, acting locally at the site of inflammation. It is likely that by targeting $CD4^+$ T cells, the pathogenic cycle of events leading to synovial inflammation and progressive joint destruction is abrogated, as other cellular players are not recruited towards the articular tissue to cause inflammation. This impact of CD4-blockade on other cell types is well illustrated by the marked decrease of pro-inflammatory cytokines produced by DCs and macrophages in anti-CD4 treated animals. In addition, given Th17 cells have been described as involved in the production of auto-antibodies in experimental autoimmune arthritis [346,347], our observation that anti-CD4-treated mice do not produce RF is in line with the hypothesis that by maintaining pathogenic T cell clones under control the B cells will not receive the necessary stimuli for the production of auto-antibodies. We cannot exclude, at this time, a direct effect of non-depleting anti-CD4 MAbs on innate cells expressing CD4, namely NKT cells that have been reported as being able to provide "help" to B cells, and to influence autoimmune arthritis [348].

In our experiments, we found a limited efficacy for tolerance induction once the animals became overtly arthritic. Although we do not have a complete explanation for this observation, it is possible that such resistance to tolerance induction may be due to the participation of other cell types, besides $CD4^+$ T cells, at that late time. In fact, in transplantation it is known that in pre-sensitized animals a population of Asialo $GM1^+$ $CD8^+$ T cells can create a barrier for tolerance induction with MAbs [349]. Thus, it may be possible to enhance the efficacy of tolerance induction in overt arthritis with reagents targeting other cell types, namely $CD8^+$ T cells and B cells.

It remains to be established whether the long-term protection from arthritis afforded following anti-CD4 treatment, even after a new curdlan challenge at a later time, can be explained by the development of regulatory mechanisms that have been described in other animal models of anti-CD4 induced immune tolerance [275,350]. In fact, it is now established that $CD4^+$ T cell activation in the presence of TGF-β and IL-6 favors T cell conversion towards arthritogenic Th17 cells, while activation in the same environment devoid of IL-6 shifts the differentiation from Th17 towards $Foxp3^+$ Treg cells [57]. SKG autoimmune arthritis development is known to be dependent on Th17 [309].

Our data suggests that protection from arthritis induced with anti-CD4 is associated

with an overall decrease of infiltrating T cells in synovial tissue and, within those T cells in the synovium, with an increase in the frequency of synovial Foxp3$^+$ Treg cells. As a consequence, the tissue is endowed with local changes that are likely to prevent the onset of arthritis directly within the local environment where inflammation would occur, even following a later exposure to curdlan at a time (day 60) where anti-CD4 MAbs are no longer present. Moreover, the reciprocal decrease of IL-17 expression at the same time that the expression of Foxp3 increases, in the joints of anti-CD4 treated mice, supports the hypothesis that indeed the balance between Treg and Th17 can determine the decision between prevention or onset of autoimmune arthritis. The observation that IL-6 decreases in the serum of anti-CD4 treated mice is also in agreement with this hypothesis. It should be noted, however, that the number of T cells is markedly reduced in the synovium of treated mice, as can be seen in the histological sections (and confirmed with greater CD3 expression in the synovia by RT-PCR – thus the need to use CD3, rather than a housekeeping gene, to normalize our gene expression studies). It was recently reported that IL-17A can be produced by mast cells in rheumatoid arthritis synovia [351]. Although we did not investigate this possible source of IL-17, the overall quantification of IL-17 transcripts was significantly reduced in anti-CD4 treated animals, where T cells were also less abundant. As a consequence, the shift in the Treg/Th17 balance in the synovial tissue is likely to be also influenced by tissue accessibility to different types of effector T cells.

We have also shown that CD4-blockade can directly inhibit T cell polarization towards an IL-17-producing Th17 phenotype, even when the most appropriate cytokine environment is provided. In addition, our data show that even in the presence of those cytokines known to inhibit Foxp3 induction (namely IL-6), CD4-blockade can facilitate the peripheral conversion of Foxp3$^+$ Treg cells. Taken together, our data complements previous studies on the mechanism of tolerance induction in transplantation with anti-CD4 (where peripheral induction of Foxp3$^+$ Treg cells have been shown critical), by demonstrating that CD4-blockade can also lead to a direct inhibition of Th17 polarization – a critical factor for the arthritis pathogenesis, namely in SKG mice [309].

Given the essential role of CD4$^+$ T cells in the pathogenesis of RA, both directly and by recruiting and activating other participating cell types (such as B cells, DCs and macrophages), the therapeutic targeting of CD4$^+$ lymphocytes has been extensively pursued [352]. Depleting and non-depleting anti-CD4 MAbs have been tested in several animal models of autoimmune arthritis [262,323,353,354], and in clinical trials with RA patients [355-357]. In spite of promising results in pre-clinical studies, the therapeutic effectiveness of anti-CD4 in clinical trials was modest and short-term, possibly due to transient immunosuppression and not tolerance. In retrospect, those unimpressive results are not

too surprising due to technical details related with dosing and MAb characteristics. In fact the immunogenicity of the mouse or chimeric MAb used was well documented as leading to their rapid clearance and consequent loss of efficient CD4 blockade [358]. The reduction of the number of $CD4^+$ T cells was also associated with a concern of possible increased susceptibility to infection.

Our data show that non-depleting anti-CD4 can be effective in preventing chronic autoimmune arthritis while preserving immune competence, as treated mice remain able to mount a CD4-dependent immune response against a different antigen (OVA). OVA immunization is a well established protocol for the induction of CD4-dependent production of OVA-specific Igs. Moreover, administration of anti-CD4 together with OVA prevents the effectiveness of subsequent immunizations with the same antigen. Taken together, these data suggest that anti-CD4 prevents immune responses towards antigens present at the time of treatment, without hampering immune responses to unrelated antigens introduced in the organism at a later time, therefore preserving immunocompetence. It should be noted that these types of studies, on the long-term immunocompetence of treated mice have been difficult to perform in animal models that, unlike SKG mice, do not develop a chronic form of arthritis.

In summary, we show that therapeutic strategies leading to synovial accumulation of Treg cells and reduction of Th17 are capable of protecting from the onset of autoimmune arthritis, as well as to prevent long-term disease progression, without leading to overall immune suppression.

Multiple Sclerosis

3. Multiple Sclerosis

3.1. Background

MS is a chronic neurological autoimmune disease characterized by inflammation, demyelination, axonal injury, and atrophy in human CNS. It is the most common neurological disease, affecting more than 1 million people worldwide (with 350000 affected individuals in North America and 500000 in Europe[359]), mainly young adults. There are twice as many females with MS than males[360]. In face of an increasing prevalence, a chronic disease course, and the partial effect of current disease-modifying drugs, MS has major social and economical consequences. The distribution of MS worldwide reflects a genetic predisposition to disease associated with a genotype encoded by the HLA locus on chromosome 6. Individuals with a particular HLA type (HLA DR2) are three to four times more susceptible to MS[361]. Moreover, several studies have reported an additional association between certain polymorphisms in CTLA-4 gene and MS susceptibility[362,363].

MS is a complex disease involving different immunopathological processes leading to an extensive heterogeneity in clinical manifestations, disease course (that include relapsing-remitting and progressive forms), and detailed pathological features among patients. This heterogeneity suggests that different pathways and effector mechanisms can lead to chronic autoimmune disease in the CNS[364]. Neuropathologically, CNS tissue from MS patients shows discrete lesions (predominantly in the white matter) with inflammatory infiltrates, demyelination, astrogliosis, ODCs apoptosis and early axonal damage. Autoimmunity is thought to result from severe inflammation driven by T cells specific for self-antigens expressed in the myelin sheath[365]. The initiation of CNS inflammation requires peripheral activation of myelin-specific T cells, that gain access to the CNS through expression of adhesion molecules and chemokine receptors[366]. These T cells are then reactivated in the CNS by APCs presenting self-antigen. There are a number of cells within the CNS capable of presenting antigen through MHC class II, such as astrocytes, microglial cells, perivascular macrophages and DCs. T cell activation then triggers the recruitment of innate immune cells, such as macrophages, which have important roles in mediating demyelination and axonal damage. Adhesion molecules, MHC molecules, cytokines, chemokines, NO production, and metalloproteases are all critical participants in the development of the inflammatory response in the CNS[367].

Activated B cells can also cross the blood brain barrier (BBB), infiltrate the perivascular space, and produce Igs.

The evidence for a central involvement of CNS auto-antigen-specific T cells in the pathogenesis of MS, has led to studies using an animal model of the disease named EAE (reviewed in[359]).

3.1.1. T cells in Multiple Sclerosis

Inflammatory lesions in CNS are largely populated by macrophages and lymphocytes. These lymphocytes are thought to damage both ODCs and axons resulting in demyelination and neuronal dysfunction. Although there is a consensus that MS is a T-cell mediated autoimmune disease, it is still not yet clear how auto-reactive T cells become activated and why inflammation can recur over time.

Central tolerance has been historically proposed as a major mechanism maintaining self-tolerance, a concept that has been recently revised due to the contribution of additional mechanisms such as regulatory T cells. Several studies have compared the frequency of myelin-specific T cells in MS patients and healthy controls, without the identification of any significant differences, although MS patients tend to have more T cells with a pro-inflammatory phenotype, as well as different epitope specificities, suggesting the presence of higher avidity T cells[368]. A higher frequency of T cells expressing degenerate TCRs in MS patients was also reported[369].

Both $CD4^+$ and $CD8^+$ T cells are present in MS lesions, with CD4+ T cells being found predominantly in the perivascular cuff and CD8+ T cells in the center and border of the lesion[370]. It has been speculated $CD4^+$ T cells have a role in initiating MS lesions, while amplification of damage is mediated by $CD8^+$ T cells. It is also unclear how myelin-specific T cells become activated in the periphery, because myelin proteins are exclusively synthesized by ODCs present in the CNS. However, TCR-transgenic mice with T cells specific for myelin peptides, like proteolipid protein (PLP) or MBP, develop spontaneous EAE. In these mice the TCR-transgenic T cells are activated in CNS-draining lymph nodes, suggesting that some myelin antigens have access to cervical lymph nodes and under certain conditions trigger T cell activation[371,372]. Another hypothesis is that myelin-specific T cells cross-react with epitopes from infectious agents and become inappropriately activated (molecular mimicry)[373]. Several studies have described degenerate TCRs which recognize both myelin antigens and pathogens-derived epitopes[373-375], even though, no pathogens have yet been identified as triggers for MS. Once self-tolerance is broken, the repeated release of self antigens from the brain allows activation of more auto-reactive T

cells responding to additional myelin epitopes. This process, termed epitope spreading, is crucial in chronic EAE models[376].

MBP and PLP are the most abundant proteins in the myelin sheath and were the first identified as $CD4^+$ T cell targets in EAE. Additionally, some mouse strains were shown susceptible to EAE when immunized with less abundant proteins, like myelin oligodendrocyte glycoprotein (MOG). This EAE susceptibility was associated to certain MHC haplotypes. It was initially thought that $CD4^+$ T cells mediating autoimmunity had a Th1 phenotype characterized by IFN-γ production. However, Th1 role in the pathogenesis of EAE has been recently questioned. Several studies have shown that IFN-γ KO as well as TNF KO mice, developed a more severe form of EAE than WT mice[377,378]. Conversely, genetic deficiency of IL-17, or neutralization of IL-17 in mice, leads to less severe EAE, although without complete protection from the disease[207,379]. Given that type I IFN induction pathway constrains Th17-mediated CNS autoimmune inflammation in mice[380], the two observations discussed above are consistent. Furthermore, mice deficient in IL-23, an important cytokine for Th17 stability, are not susceptible to EAE, whereas IL-12-deficient mice (important for Th1 commitment) show greater susceptibility to the disease[58]. Finally, IL-6 blockade in mice, a cytokine required for Th17 polarization, inhibits EAE development[381].

The role of Th17 cells was also confirmed in MS patients, with an important role for IL-17 in MS lesions[208,382] and IL-17 production by CNS-infiltrating T-cells being associated with BBB disruption and disease activity[383]. Curiously, according to a recent report, Th17 cells can enter non-inflamed brain via the choroid plexus through CCR6-CCL20 interactions[384]. Also in agreement with a role of Th17 cells in MS is the observation that IL-23 is increased in myeloid DCs from MS patients[385].

The hypothesis that MS, as well as other autoimmune diseases, is closely linked to defects in immune regulation has been extensively investigated. MS patients show no deficit in the number of circulating Treg cells, identified as $CD4^+$ $CD25^+$ T cells[386,387]. However, Tregs from the peripheral blood of relapsing-remitting MS patients display reduced *in vitro* suppressive activity, found to be intrinsic to Tregs and not due to higher resistance of conventional T cells to suppression[387-389]. This functional defect is associated with reduced Foxp3 expression[388]. The functional deficit in Tregs from MS patients has been correlated with age[389] and with disease duration[386].

The potential of Tregs to control CNS autoimmunity has been well documented in several experimental models. Studies in MOG-induced EAE, have shown Tregs accumulate within the CNS during recovery phase, and importantly this was only seen in CNS[390]. These Tregs were also shown to be highly suppressive *in vitro,* and their adoptive transfer

reduced EAE severity *in vivo*. The *in vitro* suppressive effect could be partially abrogated by neutralization of IL-10 receptor, or with Treg cells from IL-10 deficient mice, suggesting a crucial role for this cytokine in Treg-mediated EAE suppression[391]. Moreover, transfer of large numbers of polyclonal Tregs from unimmunized mice allowed disease protection in both C57Bl/6[392] and SJL recipients[391]. Conversely, Treg depletion prior to immunization increased disease severity[390,393,394], correlating with enhanced IFN-γ, IL-6, and IL-17 production[395], and recovery phase in WT mice was delayed or abrogated[390,396]. Similarly, in spontaneous animal models of EAE, such as the MBP-specific TCR-transgenic $RAG^{-/-}$ mice (TR-), the transfer of polyclonal $CD4^+$ or $CD4^+$ $CD25^+$ T cells from WT animals greatly reduced disease incidence and severity[397,398]. Post-recovery suppressor $CD4^+$ T cells were shown to inhibit the production of IFN-γ in co-culture with effector T cells, and this was specific to MBP stimulation but not other antigens[399,400]. Data from different mouse strains suggests that the higher the specificity of the Tregs to the target self-antigen, the higher protection against autoimmunity development. The generation of Tregs with specificity to the self-antigen requires its expression in the thymus, for their normal development and maintenance[401]. For instance, SJL mice higher susceptibility to EAE is related to a lower frequency of Tregs specific for PLP, and a lower thymic expression of PLP in SJL mice[402]. In resistant mice, such as B10.S strain, a greater proportion of $PLP_{139-151}$-reactive cells are within $CD25^+$ subpopulation, and the depletion of this population rendered them susceptible to actively-induced EAE[402]. Moreover other important findings, in passive EAE, suggest that the accumulation/activation of disease-relevant Tregs occurs in the CNS, and it is not necessarily mirrored in the peripheral organs, such as cervical lymph nodes[403]. Therefore, when testing regulatory T cells from human disease, it is likely that the Tregs present at the site of inflammation have greater suppressive function than their counterparts in the peripheral blood. In agreement, a human study showed enhanced Foxp3 expression in the cerebrospinal fluid of MS patients[404]. However, Tregs accumulation at the site of inflammation may be ineffective to modulate Th17 provoked disease due to the surrounding inflammatory environment[183].

Overall, if Th17 are the main mediators of autoimmune pathogenesis in MS, and since these cells and regulatory T cells have reciprocal developmental pathways, it is probably the environmental conditions, and the balance between each subset that will dictate the outcome of the disease.

3.1.2. Animal Models

EAE is a useful model of acute autoimmune demyelinating disease. Some forms of EAE reflect chronic demyelination with exacerbations and remissions characteristic of MS. It recapitulates human disease at several levels, with some key clinical and histological features, namely the presence of perivascular leucocytic infiltrates, and importantly shows both hallmarks of neuronal damage occurring in early disease stages: axonal transaction and neuronal death[405,406]. Moreover, females are overwhelmingly more susceptible to EAE induction in mice, in line with the gender biased observed in MS patients. Clinically EAE is characterized by ascending paralysis, starting with tail and hind limb weakness, that commonly progresses to hind paralysis and forelimbs. The paralytic episodes vary from model to model, and can be monophasic, acute or chronic, or chronic relapsing-remitting, depending on the antigen and strain used. Therefore, and in spite of some distinctive features, EAE is a suitable model to dissect molecular mechanisms of the autoimmune inflammatory response, and hence test new therapies for MS.

EAE can be induced by immunization of susceptible animals with a number of myelin antigens including MBP[407]; PLP[408-410]; and MOG[411]. MOG, although a minor component of the myelin sheath, has been shown to be a potent encephalitogenic protein, that induces EAE in many strains and species of experimental animals[412-415]. This protein has been also implicated in the pathogenesis of MS[416,417]. In EAE, the identity of the target auto-antigen, determines the disease characteristics, including the pattern of lesion distribution in the CNS. For example, immune responses to MBP or PLP lead to lesions predominantly located in the spinal cord, whereas immunization with MOG generates both optic nerve and spinal cord lesions[418]. T cell and antibody responses to various myelin proteins can be detected in the cerebrospinal fluid of animals with EAE. T cell immune responses spread to different portions of a given myelin protein, and then to different myelin antigens, a phenomenon known as epitope spreading. Pro-inflammatory molecules which characterize MS lesions, such as α4-integrin, matrix metalloproteases and cytokines (IL-6, TNF, IFN-γ), as well as myelin-specific $CD4^+$ T cells are also found in inflamed brain and spinal cord in EAE.

Experiments designed to investigate pathogenetic, diagnostic and therapeutic aspects of MS, taking advantage of the animal model EAE, date back to the 1^{st} half of the 20^{th} century[419]. EAE has been established and studied in non-human primates, and various other species including mice and rats[420,421]. However, disease characteristics, including histopathology and clinical course, varied significantly depending on the genetic

background, source of antigenic material, and route of antigen delivery, reflecting the heterogeneity observed in the human counterpart. Nowadays, the most used EAE models are: i) *active EAE* – induced by subcutaneous injection of a myelin peptide (most commonly MBP, MOG or PLP) which are emulsified in complete Freund's adjuvant (CFA), containing mineral oil and *Mycobaterium tuberculosis*, followed by intravenous injection of pertussis toxin; ii) *passive EAE* – intravenous adoptive transfer of myelin-reactive CD4+ T cells pre-activated *in vitro* (Th1 or Th17 differentiated lymphocytes); and iii) spontaneous TCR-transgenic models, consisting in mice with a monoclonal population of T cells bearing transgenic TCR specific to a myelin antigen.

TR- mice

EAE spontaneously develop in 100% of mice harboring a monoclonal MBP (Ac1-11)-specific $CD4^+$ αβ TCR[38]. This was achieved by crossing MBP-specific $CD4^+$ αβ TCR transgenic mice with $RAG^{-/-}$ animals. The development of spontaneous disease in this model can be prevented by adoptive transfer of $CD4^+$ splenocytes or Treg cells.

2D2 mice

The generation of MOG_{35-55} specific- TCR transgenic mice, named 2D2 mice, was first described by Bettelli et al[422]. These mice were shown to develop spontaneous optic neuritis, which was never described in other TCR transgenic EAE models. Optical neuritis is a common initial sign in 30-60% patients with MS[423,424]. Upon immunization with MOG peptide and pertussis toxin, these mice develop EAE. T cells from 2D2 mice can be transferred into C57Bl/6 WT mice, where they can lead to disease following immunization.

Pertussis toxin

It is known to promote permeabilization of the blood brain barrier, maturation of APCs[425], activation of CNS auto-antigen-specific T cells, including Th17 cells, and alteration of lymphocyte migration[426]. TLR-4 has been suggested as the critical PRR involved[427]. Interestingly, pertussis toxin has been shown to selectively reduce the number and function of Foxp3+ Tregs *in vivo*[393,428]. Moreover, CD25 depletion prior to EAE induction, has been shown to overcome the need for pertussis toxin[394].

3.1.3. Therapeutic strategies

The therapeutic options for treatment of MS have improved tremendously in the past decade. However, a treatment regime able to restore the healthy state of immune tolerance remains a critical unmet medical need. Even though global immunosuppression was among the first approaches in an attempt to attenuate the immune response in relapsing-remitting patients, there has always been a concern to target specific molecules in order to reduce the adverse effects of global immunosuppression. The use of mAbs allows targeting specific molecules involved in disease pathogenesis. Natalizumab was the first mAb approved for clinical application in MS, being specific to α4-β1integrin (VLA-4), thus reducing leucocyte infiltration into the CNS[429]. Alemtuzumab (or Campath-1H) targeting CD52 on T cells was also shown promising on ongoing clinical trials[430]. Other frequently used therapies include immunomodulation with IFN-β and galtiramer acetate (GA). IFN-β curtails T-cell trafficking, exploits the old paradigm of immune deviation towards a Th2 immune response[431], and it exhibits antiviral properties. GA is a synthetic polypeptide composed of MBP aminoacids. It is believed to inhibit monocyte activity thus inducing bystander immunosuppression at lesion sites in the CNS. It might also promote neuro-regeneration, because GA-reactive cells were reported to release neurotrophic factors[431].

A major concern is to find a therapy that significantly delays long term disease. Several mAbs were tested in clinical trials, following promising results in EAE models. The potential effect of selective blockade of proinflammatory cytokines or cytokine receptors, and administration of anti-inflammatory cytokines or soluble receptors has been extensively evaluated. Although many of these therapies ameliorate EAE, none was yet brought to phase III clinical trials due to significant toxicity or lack of efficacy. CTLA4-Ig (abatacept), approved for RA treatment, was shown to be more effective as a preventive treatment than in treating established disease[432]. Other therapeutic approaches such as statins[433] which have immunomodulatory effects, or antigen specific therapies[434], have been exploited with no significant success.

There are currently three mAbs, targeting molecules of the immune system, which were shown effective in phase II and III clinical trials: rituximab, alemtuzumab and daclizumab. Alemtuzumab binds to CD52, an antigen widely expressed in the immune system, including the surface of T and B lymphocytes, NK cells and a majority of monocytes and macrophages, and was effective in improving the relapse rate of MS patients, compared to IFN-β therapy[435]. Rituximab targets CD20, expressed on B cells, and has produced a rapid reduction in acute disease assessed by MRI[436]. Daclizumab targets

CD25, expressed on activated T cells and some Tregs, and was also shown to reduce acute disease activity[437]. Back in the 90s antibodies targeting CD4 and CD3 were among the first biologics to be tested in MS, although leading to somehow disappointing results[438]. It should be noted, however, that at that time the reagents used were significantly more immunogenic than the humanized or human mAbs used today. Recent studies in EAE, with engineered non-mitogenic anti-CD3 mAbs[439] show promising results inducing long term tolerance, through the induction of Treg cells. One of the approaches involves oral administration of anti-CD3 mAb, leading to amelioration of EAE through induction of a Treg cell subset, $CD4^+$ $CD25^-$ LAP^+, functioning through a TGF-β-dependent mechanism[440]. Moreover, treatment with an anti-CD3 mAb which transiently depletes large numbers of T cells, leads to an increased production of TGF-β by phagocytes exposed to apoptotic T cells, consequently inducing a population of $Foxp3^+$ T cells leading to long-term tolerance[167]. Of note, a non-depleting anti-CD3, engineered to remove glycosylation sites important for their immune-stimulatory function, was effective in the treatment of early onset type I diabetes[441].

Therapies favoring expansion of Treg cells are potentially a good strategy to achieve long-term tolerance. Mabs targeting CD4 were shown successful in some EAE models[265,442], but clinically it was soon abandoned. Having in mind the potential of this antibody in tolerance induction in transplantation studies, and the recent data from our lab concerning its effect on the ratio Tregs/Th17 in rheumatoid arthtritis[443], we revisited the role of non-depleting anti-CD4 in different EAE models.

3.2. Material and Methods

Animals. C57Bl/6, (H^{-2u}) MBP-specific TCR transgenic mice in a RAG-$1^{-/-}$ (TR-) background (described previously[187]), 2D2 MOG-specific TCR mice[422] were bred and maintained under SPF conditions. Experimental animals were between 8-10 weeks of age and sex matched. All experiments involving animals were approved by the Animal User and Ethical Committees at the Instituto Gulbenkian de Ciência, according with directives from Direcção Geral Veterinária (PORT 1005/92). All experiments with 2D2 MOG-specific TCR mice were done at INSERM University hospital, in Toulouse, in collaboration with Doctor Lennart Mars, inserted in Roland Liblau laboratory team.

EAE induction and treatment. C57Bl/6 WT mice were immunized with 100 µg MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) (Biopolymers Laboratory of Harvard Medical School) emulsified in CFA solution (4 mg/mL of mycobacteria (Difco) in incomplete freund adjuvant (Difco)) subcutaneously (100 µg per side flank). On the day of immunization, and two days after, mice received 200 ng pertussis toxin (List Biological Laboratories, via Quadratech) in 100 µl PBS intravenously (i.v.). Disease severity was scored daily on a 5 point scale: 1- tail atony; 2- hind limb weakness; 3-hind limb paralysis; 4-quadriplegia; 5- moribund.

In some experimental groups, EAE was induced by adoptive transfer of $1x10^5$ MOG-specific cells (isolated from 2D2 mice) to C57Bl/6 mice, which were then immunized as described above, or by adoptive transfer of pre-activated Th1 MOG-specific 2D2 T cells.

Antibodies. Non-depleting anti-CD4 (YTS177) and the isotype control (YKIX302), anti-CD25 (PC61) mAbs were produced in our laboratory using Integra CL1000 flasks (IBS, Chur, Switzerland), purified by 50% ammonium sulfate precipitation, dialyzed against PBS, and purity checked by native and SDS gel electrophoresis. The hybridomas were generously provided by Professor Herman Waldmann (Oxford, UK).

Preparation of CNS mononuclear cells. Mice were perfused through the left cardiac ventricle with cold PBS. The forebrain, cerebellum and spinal cords were dissected. CNS tissue was cut into pieces and digested with Collagenase type VIII (0,2mg/ml, Sigma) in HBSS, at 37ºC for 30 minutes. Mononuclear cells were isolated by passing the tissue through a 70 µm cell strainer, followed by a Percoll (Sigma) gradient (30%) centrifugation, at 2500 rpm for 20 minutes at 22ºC. Mononuclear cells were recovered from the pellet, ressuspended and used for further analysis.

Flow Cytometry. To prevent unspecific antibody capture by the Fc receptors, cells were incubated with anti-CD16/32 (clone 2.4G2) prior to surface and intracellular staining. Cells were washed in PBS with 0.01% NaN_3, 2 % FBS. Cells were stained for flow cytometric analysis with the following fluorochrome-labeled monoclonal antibodies: CD25 AlexaFluor488 (PC61, produced and conjugated in house), CD25 PE-Cy-7 (PC61.5, eBioscience), CD4 PerCp-Cy5.5 (RM4-5, eBioscience), CD4 PB (eBioscience RM4-5), CD3 PerCp-Cy5.5 (145-2C11, Biolegend), Thy1.2 APC-Cy7 (eBioscience, 53-2.1), 3H12 Biot (produced and conjugated in house), CD4 PE (produced and conjugated in house), CD8

APC-Cy7 (eBioscience, 53-6.7), TCR Vβ11 (eBioscience, RR3-15), TCR Vα3.2 FITC (eBioscience, RR3-16), CD45.1 A700 and Biot (eBioscience, A20).

Annexin V staining was performed with Annexin V kit, purchased from ebioscience, according to manufacturer's instructions.

Intracellular Stainings. For intracellular cytokine staining, cells were isolated as described and stimulated in complete culture medium (RPMI-1640 with Glutamax, supplemented with 10% FBS, 1% hepes, 1% penicillin/streptomycin, 1% Sodium pyruvate, 0.1 % β-mercaptoethanol (Invitrogen), containing PMA (50 ng/ml, Sigma), Ionomycin (500 ng/ml, Sigma) and Brefeldin A (10 mg/mL, Sigma) at 37º C in a humidified 10% CO_2 atmosphere for 4 hours. After staining of surface markers, cells were fixed and permeabilized using Fixation and permeabilization kit from ebiosciences, according to manufacturer's instructions. IFN-γ (XMG1.2), IL-17A (ebio 17B7) and Foxp3 (FJK165) antibodies were purchased from BD and ebiosciences.

Viral Plaque Assay. Mice were infected with Murid herpesvirus-4 (MuHV-4) by intranasal inoculation of 10^4 plaque forming units (p.f.u.) in 20µl PBS under halothane anesthesia. At days 7 (peak of lytic infection) and 14 (resolution of lytic infection), lungs were recovered and frozen at -80º C. On the day of the assay, lungs were homogenized in GMEM complete media, and submitted to 10 times serial dilutions. To each dilution 5 x 10^5 BHK-21 cells were added. Viruses were let to adsorb for 1 hour in 6 well plates, at 37º C, followed by 4 days incubation, after addition of 3 ml media. At day 4 of culture, the cell monolayer was fixed with 4% formaldehyde and stained with 0,1% Toluidine blue. Viral plaques were counted with a Stemi SV6 magnifying glass (Zeiss). Virus titers were determined from the average of the number of counted viral plaques in duplicates.

Cell culture and stimulation. Spleen cells were harvested and red blood cells lysed. Splenocytes ($1x10^6$) were cultured for 3 days in 96 well plates, with complete RPMI media, and 20 µg peptide (MBP or MOG). At day 3 cells were centrifuged and supernatants recovered and kept at -80º C until cytokine quantification.

Th17 polarization. MBP-specific CD4+ T cells from TR- RAG1-/- mice were purified by magnetic separation with CD4 (L3T4) microbeads (Miltenyi Biotec, Germany). Cell purities were between 92-96%. The T cells were cultured for 5 days with bone marrow derived DCs, 10 µg/ml MBP peptide (MedProbe, Oslo, Norway), and 0, 10 and 100 µg/ml anti-CD4 (YTS177) in IMDM 5% FBS (Invitrogen), 1% Pen/Strep (GibCo), 0.1% β-Mercaptoethanol (GibCo), 1 ng/ml TGF-β (R&D systems), 20 ng/ml IL-6 (R&D systems), 10 ng/ml IL-1β (Ebiosciences), and 10 µg/ml anti-INF-γ (R46A2). At the end of the culture the cells were harvested, and processed for flow cytometry.

ELISA cytokine detection. The quantification of cytokines in the cell-culture supernatant was performed using IL-10, IFN-γ kits (Prepotech, London, UK) or IL-17 kit (R&D Systems). All assays were performed according to the manufacturer's instructions.

T cell purification and adoptive transfer. MOG-specific naïve CD62L+ CD25- CD4+ were purified from CD45.1 congenic 2D2 mice by MACS negative selection (Miltenyi Biotec), using anti-CD8 (YTS169), anti-B220 (RA3-6B2), anti-Mac1 (CI:A3-1), and anti-CD25 (7D4) mAbs followed by positive selection with anti-CD62L-coated beads, providing 80% CD4+ T cells, of which 95% expressed transgenic 2D2 TCR. $1x10^6$ cells were adoptively transferred to naïve C57Bl/6 WT mice.

CFSE staining. Cells were diluted at $1x10^6$ cell/ml in complete RPMI medium. One µl of CFSE (at 5mM) was added per ml of cells, and incubated at 37º C for 10 minutes. To stop the staining, cold complete medium was added.

Th1 polarization and adoptive transfer. CD4+ T cells were magnetically isolated from spleen and LN of 2D2 mice, ressuspended at $0.5x10^6$ cells/ml and cultured with 5–$10x10^6$ irradiated APCs/ml. Cultured cells were stimulated with 20 µg/ml MOG peptide in medium, supplemented with 10% FCD, 1ng/ml IL-2, 20ng/ml IL-12 (R&D systems). At day 6, cells were washed, isolated through a Ficoll separation gradient, and re-stimulated for another 3 days under the same conditions. At day 9, living cells were collected again by Ficoll density separation and used for adoptive transfer. We transferred $14x10^6$ Th1 cells per recipient C57Bl/6 mouse.

Histology. Animals were killed and perfused with 2% paraformaldehyde in PBS. Tissues were embedded in paraffin, and tissue sections were stained with H&E.

Statistical analysis. Statistical significance was determined using a two tailed non-parametric Student's t tests (Mann-Whitney) with Prism 4.0 (GraphPad Prism 5). Significant differences were considered when *p<0.05* (**p<0.05; **, p<0.01; ***, p<0.001*).

3.3. Results

3.3.1. Non-depleting anti-CD4 prevents the onset of EAE

Non-depleting anti-CD4 has been extensively used in transplantation studies, being able to induce Treg-mediated dominant tolerance[231]. To assess the effect of this MAb in EAE we induced the disease in C57Bl/6 WT mice through subcutaneous immunization with MOG-CFA followed by intravenous pertussis toxin. Some animals were treated with 1 mg anti-CD4 on the days -3 and -2 in relation to MOG-CFA immunization. All mice treated with anti-CD4 remained protected from EAE (Figure 1A).

To test the efficacy of anti-CD4 treatment after disease onset, mice were treated with anti-CD4 upon initial manifestations of EAE (score 1). The progression of the disease was halted in anti-CD4-treated mice, with most animals displaying full recovery (Figure 1B).

Anti-CD4 treated mice exhibited reduced inflammatory infiltrates in the CNS, including lower numbers of CD4 T cells, on day 14 following MOG-CFA immunization, down to the levels of naïve mice (Figure C). Cervical lymph nodes (CLN) from anti-CD4 treated mice have a consistent increase in the frequency of Foxp3+ Treg cells, that however does not reach statistical significance (data not shown), which may be a consequence of the small representation of antigen-specific Treg cells among Treg cells of other specificities. However, Th17 content in the CLN is significantly reduced as established by measuring IL-17 production in cultured splenocytes stimulated with MOG (thus allowing the evaluation of MOG-specific clones) (Figure 1D).

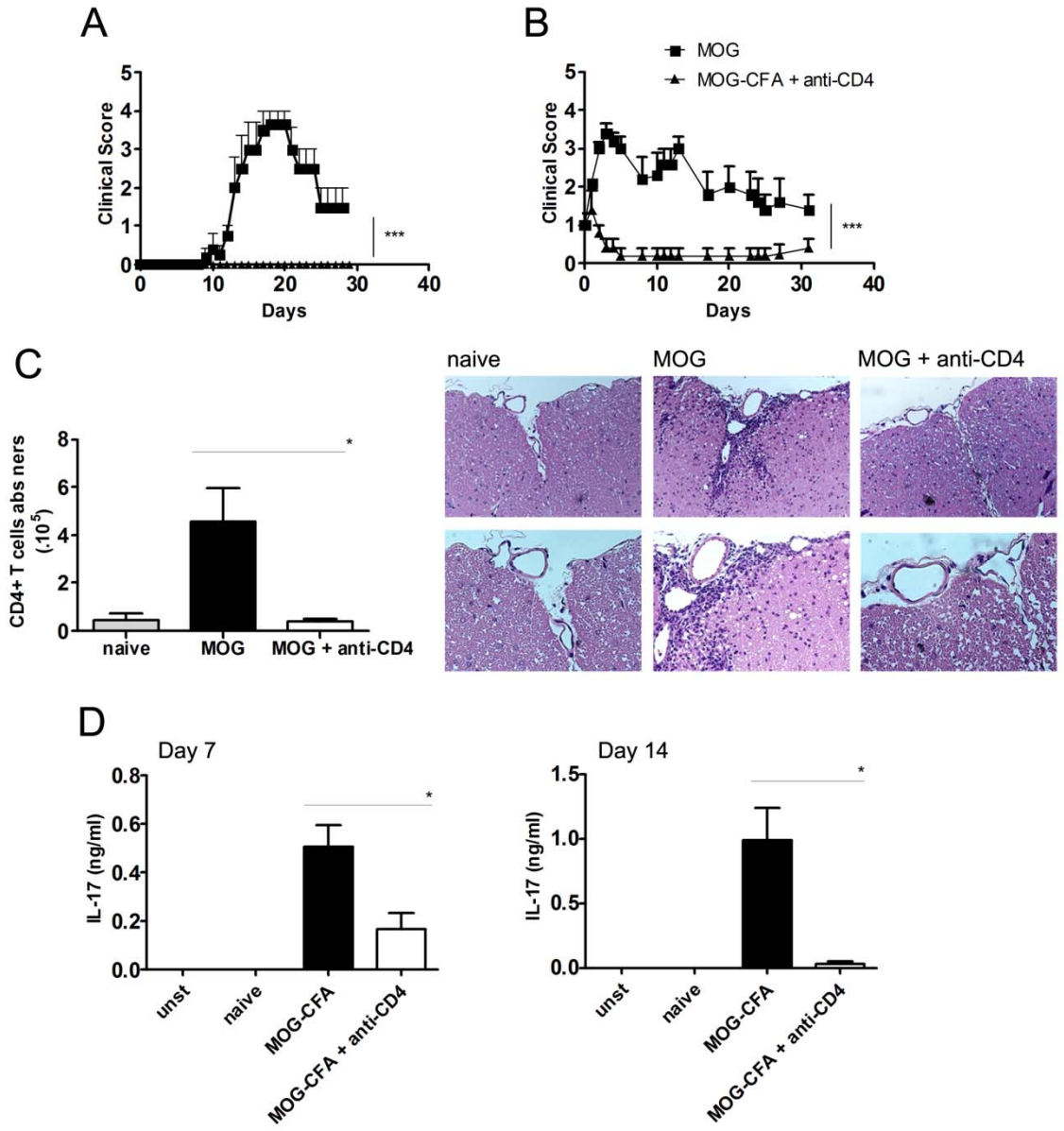


Figure 1 - Non-depleting anti-CD4 Mab prevents EAE development in C57Bl/6 mice. (A) EAE was induced in C57Bl/6 with 100µg MOG-CFA s.c.per flank and 200 ng pertussis toxin. A group of mice was treated at days -3 and -2 before immunization with 1 mg non-depleting anti-CD4 Mab. Mice treated with the Mab showed no clinical manifestations of EAE (***P<0.001). **(B)** Mice were immunized with MOG-CFA as described, with anti-CD4 treatment starting upon the initial manifestations of EAE (when clinical score reached 1). Disease progression was halted in Mab-treated mice (***P<0.001). **(C)** Number of CD4+ T cells within the CNS. Anti-CD4 treated mice had a significant reduction of CNS-infiltrating CD4 T cells, similar to naïve mice (*P<0.05). Hematoxilin & eosin staining of a transversal section of the spinal cord of naïve, MOG and anti-CD4 treated group (200x and 400x magnified). There is a marked protection from inflammatory infiltrates in anti-CD4 treated mice. **(D)** Concentration of IL-17 in supernatants of MOG-stimulated spleenocytes at day 7 and day 14 after MOG-CFA immunization. Th17 cells from MOG-CFA immunized mice, produced more IL-17 than from anti-CD4 treated mice (*P<0.05)

3.3.2. Mice treated with non-depleting anti-CD4 MAb remain immunocompetent

In order to evaluate the impact of anti-CD4 treatment in immune competence, we evaluated the clearance of a gamma-herpesvirus by mAb-treated mice. MuHV-4 causes transient pneumonia in C57Bl/6 mice, being efficiently cleared from the lungs approximately 14 days after infection. MOG-CFA immunized mice were treated with anti-CD4 as described above. On day 30 following MOG-CFA immunization and anti-CD4 treatment, the mice were intranasally infected with MuHV-4. Lungs were recovered at day 7 and day 14 post-infection, to evaluate the titer of virus infection at the peak and at the time of infection resolution. Control mice were infected with MuHV-4 in the absence of prior MOG-CFA immunization or CD4-blockade. Anti-CD4 treated mice were able to resolve the MuHV-4 virus infection as effectively as non-manipulated control mice (Figure 2). These results show anti-CD4 treated mice remain immunocompetent to mount protective immune responses.

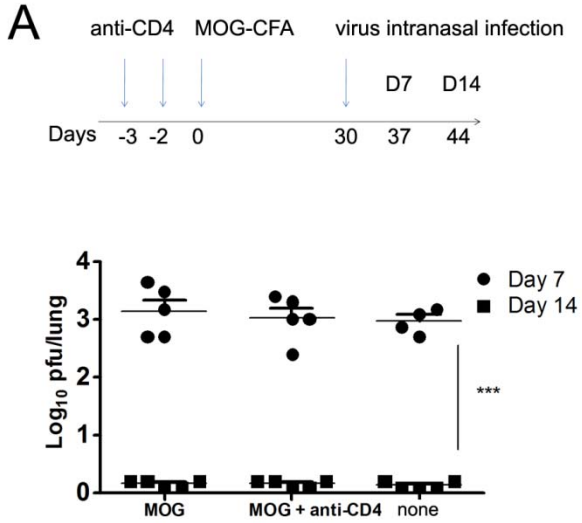


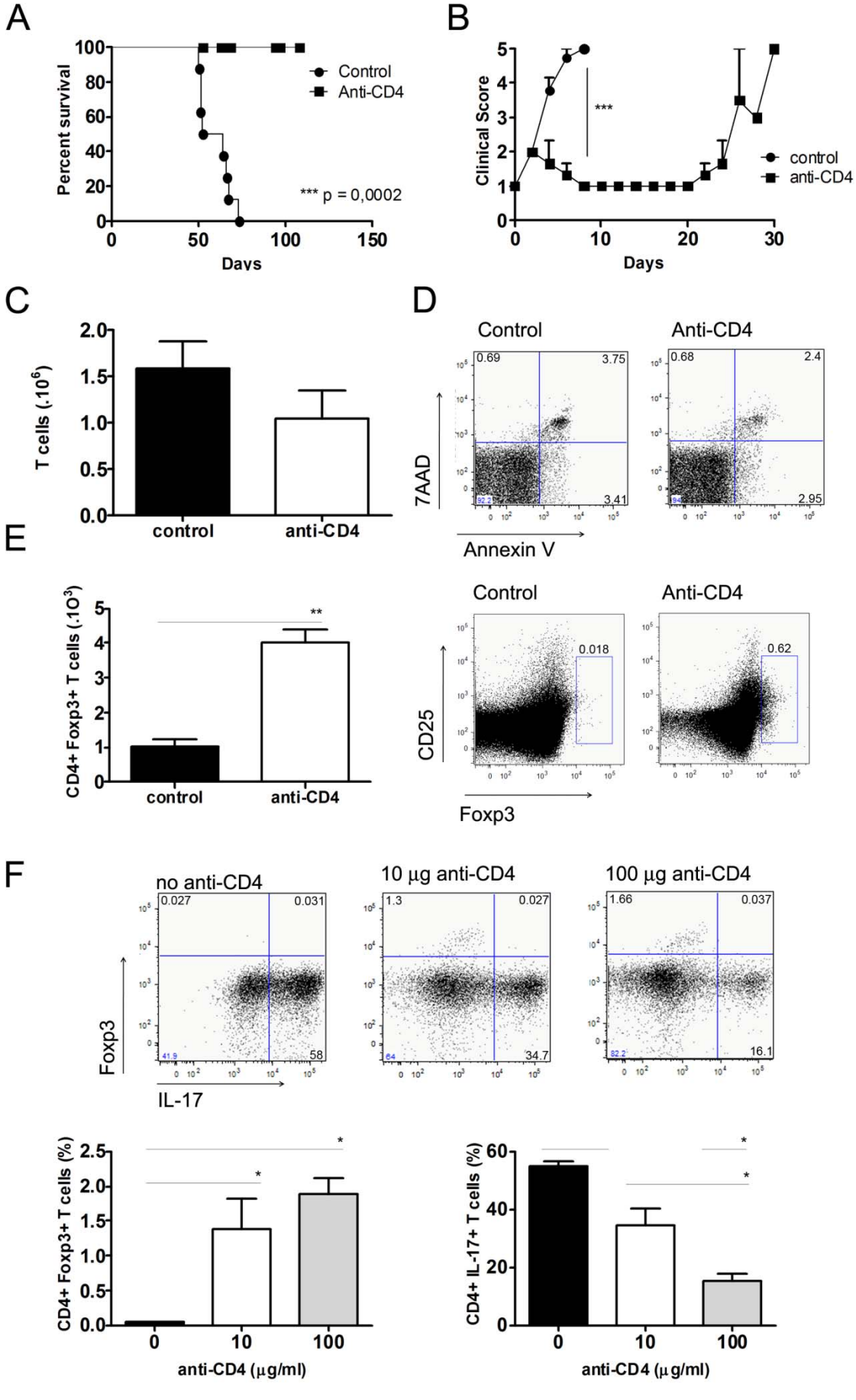
Figure 2 – Anti-CD4 treated mice remain immunocompetent – C57Bl/6 mice infected with MuHV-4 develop pneumonia which reaches its peak at day 7, being resolved by day 14 in normal immunocompetent mice. We administered 10^4 plaque forming units of MuHV-4 intranasally to mice immunized with MOG-CFA and treated with anti-CD4. The viral load was quantified from lungs collected at days 7 and 14. Anti-CD4 treated mice were as efficient as control groups in clearing the viral infection by day 14 (****P<0.001*).

3.3.3. Anti-CD4 prevents chronic fatal EAE in TCR-transgenic mice

RAG-1 deficient MBP-specific TCR-transgenic mice (TR-) spontaneously develop EAE between days 40 and 60 of life[187]. The disease progresses rapidly, leading to death within 5 days upon the initial clinical manifestations. Treatment with 2 shots of 1 mg anti-CD4 between 3rd week and 4th week of birth allowed complete protection from EAE (Figure 3A). In fact, anti-CD4 treatment allowed the maintenance of the colony has homozygotes. Moreover, when mice were treated after the onset of EAE, once clinical score reached 1, the disease progression was halted (Figure 3B).

We found that the overall CD4$^+$ T cell number is maintained following anti-CD4 treatment, confirming the non-depleting nature of the Mab (Figure 3C). In addition, we did not find evidence for increased apoptosis of cells from anti-CD4 treated mice (Figure 3D).

As TR- mice are devoid of natural Treg cells we could evaluate peripheral Treg induction in anti-CD4 treated mice. Importantly, we observed an increased number of Foxp3$^+$ T cells in anti-CD4 treated mice (Fig 3 E). Given the *in vivo* data, we evaluated whether *in vitro* stimulation of MBP-specific TR- cells with MBP loaded DCs in the presence of anti-CD4 would alter the balance between the protective and pro-inflammatory Treg and Th17 populations. Addition of anti-CD4 to cultures in Th17 polarizing conditions (in presence of TGF-β and IL-6), led to a decrease in Th17 cells with a simultaneous increase of Treg frequency, in a dose dependent way (Figure 3F).


A
Percent survival
Control
Anti-CD4
*** p = 0,0002
Days
B
Clinical Score
***
control
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Days
C
T cells (.10^6)
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anti-CD4
D
Control
Anti-CD4
7AAD
Annexin V
E
CD4+ Foxp3+ T cells (.10^3)
**
control
anti-CD4
Control
Anti-CD4
CD25
Foxp3
F
no anti-CD4
10 µg anti-CD4
100 µg anti-CD4
Foxp3
IL-17
CD4+ Foxp3+ T cells (%)
anti-CD4 (µg/ml)
CD4+ IL-17+ T cells (%)
anti-CD4 (µg/ml)

Figure 3- Anti-CD4 prevents fatal EAE in TR- mice – (A) TR- mice develop fatal spontaneous EAE between day 40 and 60 of life. We treated a group of TR- mice between days 30 and 35 with 2 shots of 1 mg anti-CD4. Treated mice were protected from the onset of EAE (***P<0.001). **(B)** To investigate the impact of anti-CD4 treatment in established disease, we treated a group of mice upon the first manifestations of the disease. CD4-treated mice were protected from severe EAE while control animals died within days**. (C)** Number of CD4 T cells in cervical LNs of anti-CD4-treated and control mice. **(D)** Frequency of apoptotic cells, identified with Annexin V and 7AAD staining, in anti-CD4-treated and control mice. **(E)** Number of Foxp3+ CD4+ T cells in anti-CD4 treated mice, as well as representative dot plots. Mice treated with anti-CD4 showed significantly increased levels of Treg cells (**P<0.01). **(F)** In vitro cultures of TR- T cells stimulated with MBP, under Th17 polarization conditions. The addition of anti-CD4 MAb led to a decrease in Th17 cells and an increase in the Foxp3+ T cells in a dose-dependent way. Bar graphs and representative dot plots.

3.3.4. Anti-CD4 inhibits proliferation and functional polarization of MOG-specific T cells

To better study the mechanism through which anti-CD4 is preventing EAE, we took advantage of the 2D2 adoptive transfer model[444], since TR- are in the H^{-2u} background[38], making it difficult to perform adoptive cell transfer experiments. Therefore we used MOG-specific 2D2 cells to follow the fate of neuropathogenic T cells *in vivo* subject to the effects of anti-CD4 MAb. When adoptively transferred into C57Bl6 WT mice, these specific cells are easily tracked by the expression of a congenic marker CD45.1.

Immunization with MOG-CFA of C57Bl6 mice, adoptively transferred with $1x10^6$ 2D2 T cells led to the development of severe EAE earlier than in the absence of transferred 2D2 cells. Anti-CD4 treatment was also efficient protecting 2D2 transferred mice from EAE (Figure 4A). We analyzed T cell populations at day 3, and day 9, in draining lymph nodes and spleen. Although, the overall CD4 population was maintained following anti-CD4 treatment, given the mAb has a non-depleting isotype, there was a significant reduction in the number of MOG-specific T cells (Figure 4B,C). We labeled 2D2 cells with CFSE prior to adoptive transfer, and found that anti-CD4 treatment was preventing proliferation of MOG-specific cells, but without inducing an increase of apoptosis (Figure 4D-F).

Besides the impact of anti-CD4 MAb in 2D2 cell proliferation, we also found the MAb treatment prevented Th1 and Th17 polarization. In fact, there was a significant reduction in IL-17^+ 2D2 cells from anti-CD4 treated mice, as well as a functional impairment for LN cells to produce IL-17 or IFN-γ following *in vitro* stimulation with MOG (Figure 4G). We could not find, however, evidence for Foxp3 induction under these experimental conditions, at these early time points.

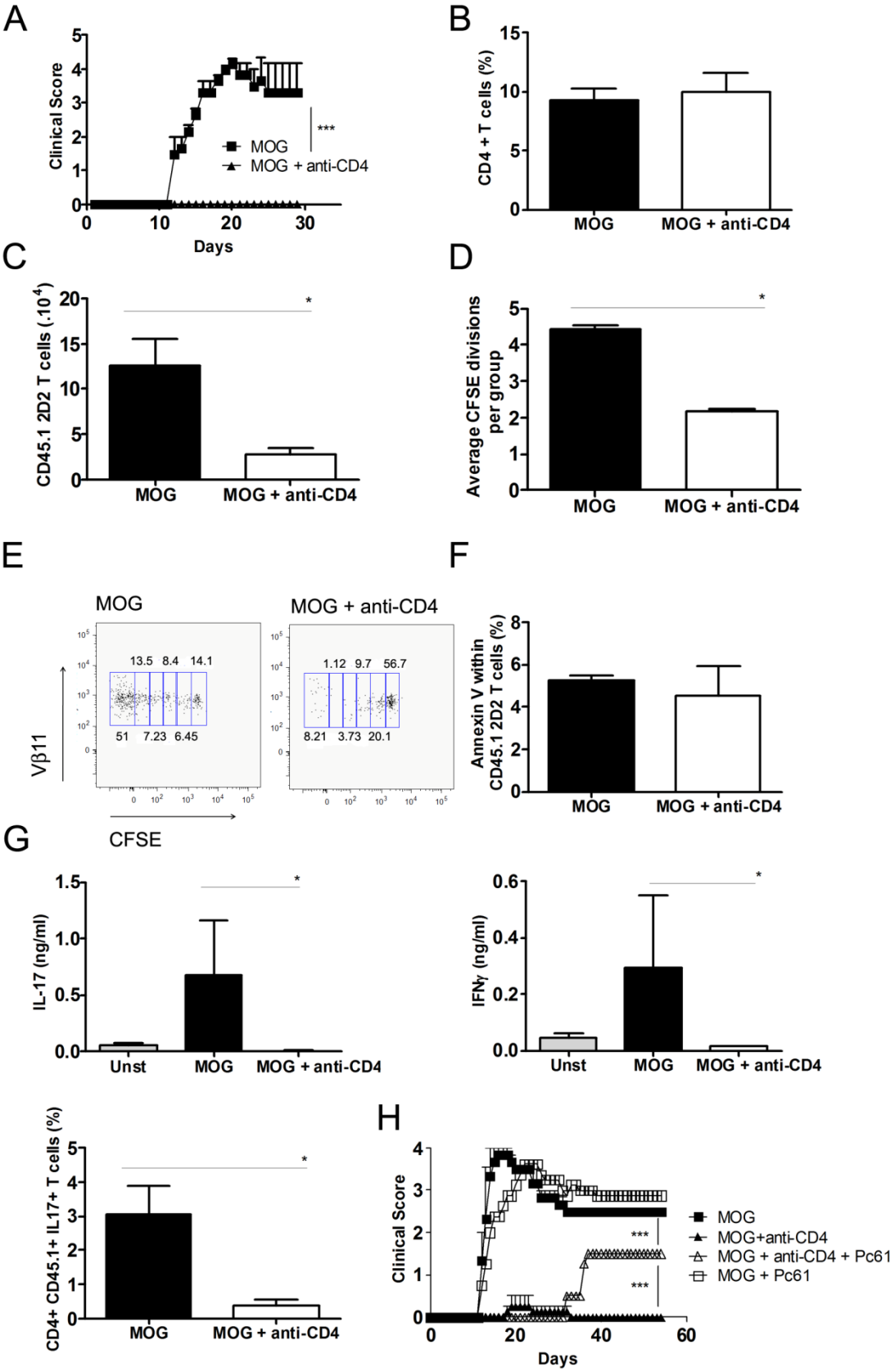
A
Clinical Score
Days
MOG
MOG + anti-CD4
***
B
CD4 + T cells (%)
MOG
MOG + anti-CD4
C
CD45.1 2D2 T cells (.10^4)
MOG
MOG + anti-CD4
*
D
Average CFSE divisions per group
MOG
MOG + anti-CD4
*
E
MOG
MOG + anti-CD4
13.5 8.4 14.1
51 7.23 6.45
1.12 9.7 56.7
8.21 3.73 20.1
Vβ11
CFSE
F
Annexin V within CD45.1 2D2 T cells (%)
MOG
MOG + anti-CD4
G
IL-17 (ng/ml)
Unst
MOG
MOG + anti-CD4
IFNγ (ng/ml)
CD4+ CD45.1+ IL17+ T cells (%)
H
Clinical Score
Days
MOG
MOG+anti-CD4
MOG + anti-CD4 + Pc61
MOG + Pc61

Figure 4 – Anti-CD4 inhibits the proliferation and differentiation of MOG-specific naïve T cells – (A) 2D2 cells were adoptively transferred into C57Bl/6 mice, subsequently immunized with MOG-CFA as described above. A group of mice was treated with anti-CD4 on days 0, 2 and 4. Treated mice remained protected from EAE (****P<0.001*). **(B)** To study proliferation of MOG-specific CD4 T cells, cells were stained with CFSE prior to adoptive transfer into C57Bl/6 mice ($1x10^6$) that were then MOG-immunized as described. At day 3, cells were analysed for cell proliferation. Anti-CD4 treatment did not affect the overall frequency of CD4+ T cells (the majority not specific to MOG) in the draining lymph nodes. **(C)** The number of MOG-specific CD4+ T cells was lower in anti-CD4 treated mice (**P<0.05*). **(D)** Average cell division (calculated based on CFSE dilution) showing MOG-specific T cells from anti-CD4 treated mice underwent less rounds of division. **(E)** Representative dot plots showing CFSE staining. Anti-CD4 treated group exhibits a higher frequency of cells with greater intensity of CFSE (undivided), while the majority of cells in the untreated group have diluted the CFSE staining (multiple divisions). **(F)** MOG-specific 2D2 cells were stained for Annexin V. No difference was found between anti-CD4 treated and MOG group. **(G)** IL-17 and IFN-γ production, evaluated in supernatants of MOG-stimulated splenocyte cultures, was greater in the MOG control group, being almost undetectable in anti-CD4 treated mice (**P<0.05*). Similarly, the frequency of MOG-specific Th17 cells was increased in animals not subjected to anti-CD4 treatment. **(H)** C57Bl/6 mice were adoptively transferred with $5x10^5$ 2D2 cells and immunized with MOG. Some groups were treated with anti-CD4 and depleted of CD25+ cells with PC61. Mice treated with anti-CD4 who were also depleted with anti-CD25 did not maintain tolerance, and started developing mild EAE once the therapeutic anti-CD4 mAb was cleared from circulation (****P<0.001*).

To investigate a possible contribution of natural Treg cells following anti-CD4 treatment, we depleted $CD25^+$ T cells from C57Bl/6 mice seven days before adoptive transfer of 2D2 cells and MOG-CFA immunization. The onset of the disease in anti-CD25 treated mice was slightly delayed, possibly due to a moderate effect of PC61 on activated $CD25^+$ effector T cells (Figure 4H). However, in mice treated with non-depleting anti-CD4 we observed a late onset of a mild form of EAE (score 2), while mice treated with anti-CD4 in the absence of CD25 depletion remained protected from the disease (score 0). Together with the observation of no iTregs at day 3 and day 9, this observation suggests a role for natural Tregs in the maintenance of tolerance, rather than on tolerance onset, following anti-CD4 therapy. Moreover, all of the experimental groups, at a later time point (day 60), developed Foxp3 expressing MOG-specific T cells (data not shown).

3.3.5. Effector T cells are committed to apoptosis under anti-CD4 treatment

Given our data on the efficacy of non-depleting anti-CD4 Mab preventing the progression of EAE, we investigated the *in vivo* impact of anti-CD4 on terminally differentiated 2D2 cells. We adoptively transferred $14x10^6$ MOG-specific 2D2 cells, previously cultured *in vitro* under Th1 polarization conditions, into C57Bl/6 mice. This procedure is known to lead to severe fatal EAE even in the absence of MOG immunization[444]. We found that anti-CD4 treatment at the time of the adoptive transfer of pre-activated 2D2 cells was able to prevent the onset of EAE (Figure 5A).

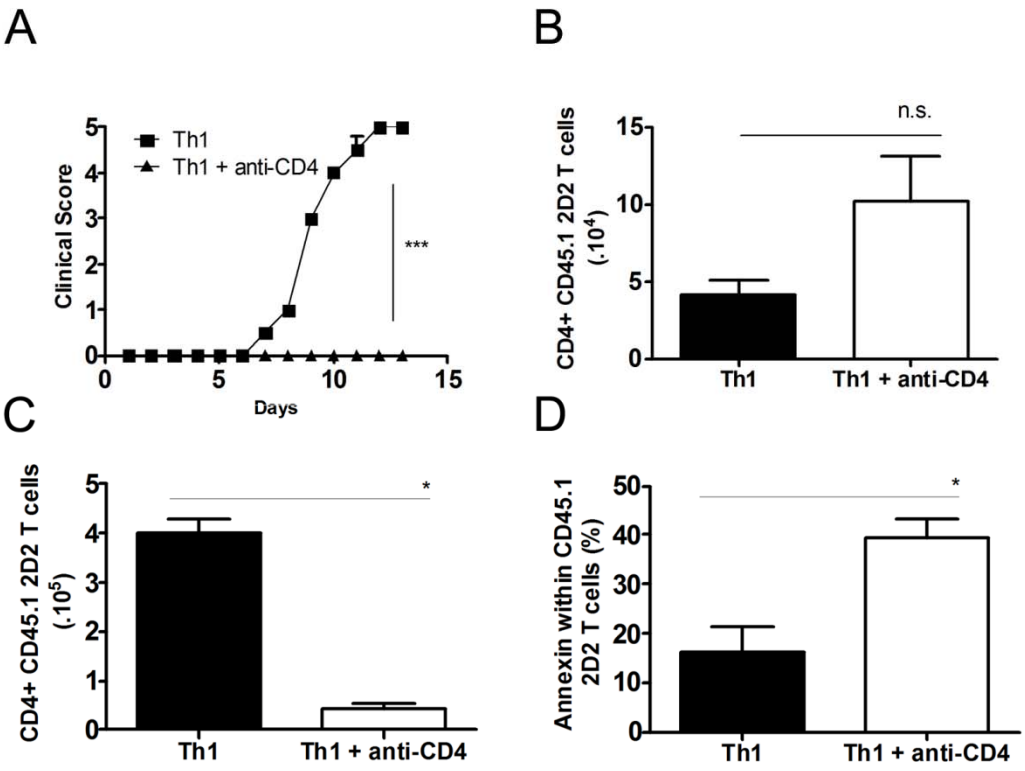

Figure 5 – Anti-CD4 prevents Th1 induced EAE through apoptosis induction - (A) MOG-specific 2D2 T cells were polarized towards Th1 phenotype in vitro, and transferred ($14x10^6$/mouse) into C57Bl6 mice. Some mice were treated with anti-CD4 at the day of Th1 transfer and at days 2 and 4. Mice transferred with Th1 cells died after 10 days of cell transfer, whereas anti-CD4 treated mice remained healthy (****P<0.001*). **(B)** Number of MOG-specific 2D2 cells in the CLNs. Anti-CD4-treated mice had more MOG-specific T cells in CLN, but MOG-specific T cells were prevented from entering the CNS of these mice **(C)**, (**P<0.05*). **(D)** Frequency of MOG-specific 2D2 cells positive for annexin V in the CLN. Anti-CD4 treated mice exhibited a higher frequency of apoptotic (annexin V+) cells (**P<0.05*).

In a different group of animals, we investigated the fate of the transferred 2D2 cells at day 9. We found no change in the number of 2D2 cells in the draining CLN (in fact there is an increase in the number of 2D2 cells in anti-CD4 treated mice that does not reach statistical significance, Figure 5B). However, there was a significant reduction in CNS infiltrating 2D2 cells in MAb-treated mice (Figure 5C). In addition, we found an increase of 2D2 cells being committed to apoptosis, as evidenced by annexin V staining, in the CLN of anti-CD4 treated mice (Figure 5D).

Taken together, our data suggest that anti-CD4 treatment acts differently in naive and fully polarized antigen-specific cells: while it prevents naive cells from proliferating and acquiring a Th1 or Th17 functional profile, CD4 blockade drives terminally differentiated T cells to apoptosis thus preventing target organ destruction.

3.4. Discussion

The availability of different TCR-transgenic animal models of autoimmune demyelinating disease allows the study of tolerance-inducing regimens and its influence in the different components of the immune system. The last decade several studies have supported a role for depleting[167,440] and non-mitogenic[439,445] anti-CD3 mAb in long-term protection from EAE. We used a non-depleting anti-CD4 mAb, and found that CD4-blockade can lead to long-term protection from EAE, while inducing different outcome in naive or pre-activated effector T cells.

We found CD4-blockade was able to prevent the onset of EAE in MOG-CFA immunized mice, as well as to impair disease progression when anti-CD4 was administered following the onset of clinical manifestations of the disease. Importantly, anti-CD4-treated mice remained fully competent to respond to unrelated antigens, or to mount protective immune responses towards a gamma herpes virus (MuHV-4). In fact, there were some promising studies with anti-CD4 in EAE protection[442,446], in the early 90's, which led it to be tested in clinical trials, however, soon this mAb was suspended due to adverse effects related to CD4 T cell depletion[438,447]. Our data show that, similarly to what was previously shown in transplantation[163,247], the tolerogenic effect mediated by anti-CD4 mAb does not require direct T cell depletion.

We could therefore use TCR-transgenic models of EAE to identify what was the mechanism leading to long term protection from EAE. First we used RAG1-deficient mice bearing a T cell repertoire exclusively composed by MBP-specific T cells and devoid of $Foxp3^+$ Treg cells[187]. In spite of the number of potentially aggressive cells these animals were protected from the development of EAE following a short course of non-depleting

anti-CD4. In fact, following such treatment it becomes possible to maintain a colony of homozygous TR- mice (data not shown). We found protection from EAE correlated with the emergence of Foxp3$^+$ Treg cells in the periphery. This is in agreement with several studies that support a role for Tregs in the control of CNS inflammation. In fact, some studies have shown that upon Tregs adoptive transfer from TR$^+$ mice, TR- remain protected from disease development[397,398]. Our *in vitro* studies have confirmed that activation of TCR-transgenic T cells in presence of anti-CD4, under conditions favoring Th17 differentiation, favor a decrease of IL-17 producing cells concomitantly with a greater frequency of Foxp3$^+$ cells in a dose dependent way.

In order to follow the fate of pathogenic T cells *in vivo* we adoptively transferred MOG-specific cells from 2D2 mice[422]. It is well described that in EAE, the CNS suffers a massive infiltration by activated lymphocytes, namely CD4 effector T cells, which break the blood brain barrier and recruit other pro-inflammatory cells to the site of inflammation, leading to an increased severity of the disease[371]. We found that protected mice did not show any significant infiltrates in the CNS as well as a reduction of MOG-specific TCR transgenic cells in the CLN (the number of non-transgenic CD4 cells did not change, confirming the non-depleting nature of the mAb used). We also found CD4 blockade was acting by preventing T cell proliferation, as well as preventing the differentiation of effector cells producing pro-inflammatory cytokines. Induced Treg cells did not appear in the initial period following treatment, being especially important for the long term maintenance of the tolerance state, something we have confirmed following depletion of CD25$^+$ T cells. This observation is consistent with the reported ability of T cells to convert to Treg when exposed to antigen for a period of time under non-inflammatory conditions[113,115,275,448,449].

We then studied the effect of CD4-blockade on pre-activated MOG-specific T cells, generally considered difficult to regulate. In fact, it was previously reported MOG-specific Foxp3 Treg cells are able to control naïve neurotropic T cells but not activated effector T cells[183]. We found pre-activated 2D2 cells do not cause pathology in presence of CD4-blockade. Our data show pre-activated 2D2 cells under the cover of anti-CD4 are prevented from infiltrating the CNS, while being committed to apoptosis.

Taken together our data show that T cells under distinct stages of functional maturation respond differently to tolerance inducing protocols. Therefore, it is likely that ideal reagents to induce tolerance in naive T cells may be inadequate to effectively control pre-activated T cells, as they are present following the onset of the disease. Similarly, strategies that can suppress terminally differentiated effector cells may be insufficient to induce long term protection from the disease by promoting the peripheral

induction of Treg cells. In a similar way to what we have previously reported in an animal model of autoimmune arthritis[443], it appears that the key to long-term tolerance in EAE is the resetting of the deregulated Th17/Treg ratio. Importantly, naive and effector cells appear to be regulated differently: CD4-blockade prevents the proliferation and Th17 polarization of naive T cells, while favoring Treg conversion; however, activated T cells are regulated by being predominantly committed to apoptosis.

Overall our data show that effective tolerance-inducing strategies in autoimmunity will have to induce naive cells to acquire regulatory function important for the long-term maintenance of tolerance, while simultaneously disarming the pre-activated effector T cells that are resistant to Treg-mediated regulation.

Anaphylaxis

4. Anaphylaxis

4.1. Background

Typically the term anaphylaxis connotes an immunologically–mediated event that occurs after exposure to certain foreign substances. It is an acute, life threatening, allergic reaction that occurs massively and systemically. This reaction results from the generation and release of a variety of potent biologically active mediators and their concerted effects on various target organs. Anaphylaxis is recognized by cutaneous, respiratory, cardiovascular, and gastrointestinal signs and symptoms occurring singly or in combination. The global prevalence of anaphylaxis is unknown, since there is no requirement to report it every time it happens. Allergic reactions to food were found to be the most common cause of anaphylactic reactions outside the hospitals, more frequent than bee stings and drugs combined. Peanuts and other tree nuts are the most common food causing serious anaphylactic reactions, and constitute a major public health problem because of its severity, persistence through adulthood, and prevalence, estimated to be between 0.5 and 1% in North America[450]. Studies on peanut allergy in the US and UK indicate that the number of children affected has doubled in the last 10 years[451]. Besides, peanut allergy accounts for 80% of food-induced fatal anaphylaxis cases, reviewed in Moneret-Vautrin et al[452]. Previous studies suggest more than one peanut characteristic contributes to its allergenicity. For example, dominant allergens in peanut include Ara h 1, Ara h 2 and Ara h 3, which are most likely related to severe clinical reactions[453]. Furthermore, roasting, which is used to process most peanuts consumed in western countries, increases peanut allergenicity[225]. Prevention of exposure to peanuts is managed through strict avoidance, which is compromised by the frequent use of peanuts or derivatives in food preparation, cosmetic creams, and other common products. Consequently there is an urgent unmet need for a suitable therapy able to induce specific clinical and immunological tolerance, and ultimately avoid fatal cases of anaphylaxis.

The prevalence of allergic diseases has increased in the last 10 to 15 years. One hypothesis for the increasing prevalence of Th2-associated allergic diseases - the hygiene hypothesis - is that western lifestyles have been reducing contact with environmental microorganisms thus leading to increased prevalence of allergic diseases[454]. It has been a long held assumption that such microorganisms may favor Th1 type responses, or the development of Treg responses, thus preventing Th2 type responses to allergens. Then, decreased exposure to immune stimulating infections in early childhood might be a cause for the increased prevalence of allergy. However, Th1 associated diseases have also been

rising along the years. Furthermore, populations with high rates of helminth infections, which are known to induce a Th2 response, are among those protected from allergic diseases[455]. Therefore, the decline in Treg cells as consequence of decreased exposure to infectious agents may provide a better explanation for hygiene hypothesis, and this would also explain the increased prevalence of autoimmune diseases throughout the years, with the same geographical distribution[456].

Anaphylaxis is classically mediated by histamine release in response to antigen crosslinking of IgE bound to FcRεI on mast cells, however cell-mediated mechanisms may also be involved[211]. Tolerance breakdown might be triggered by several allergens (pollen, house dust mite, bee venom, or food proteins). Studies from our lab have shown that non-depleting anti-CD4 mAb is able to induce long term tolerance in an allergic model of airways hypereactivity (agua-Doce et al unpublished data). We wanted to assess the ability of anti-CD4 mAb to induce tolerance in a model of anaphylaxis. We used a well established model of anaphylaxis that relies on the sensitization of a specific mice strain to crude peanut extract allergy.

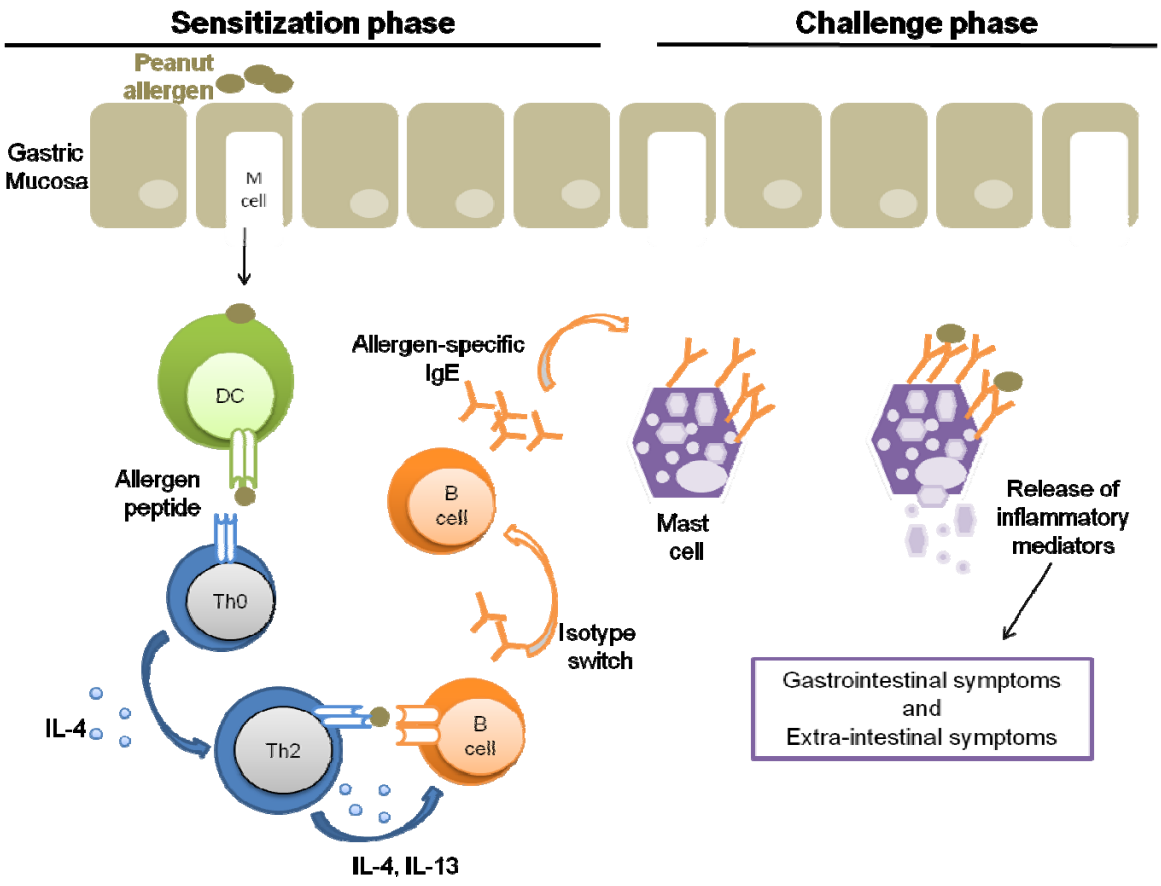

Figure 8 - Main cellular interactions during peanut allergic immune response. (A) Sensitization phase – In the gastric mucosa, peanut allergens are taken up by specialized epithelial cells called M cells and transferred to antigen-presenting cells such as DCs, being processed into peptide fragments. Complexes of peanut-allergen peptide and MHC II are presented to naïve cells, which differentiate into Th2 cells. Activated Th2 cells recognize peanut –allergen-peptide-MHC II complexes on the surface of B cells, releasing cytokines IL-4 and IL-13, which promote IgE production by B cells. Secreted IgE antibodies bind to FcεRI receptors on effector cells such as mast cells, which become sensitized. **(B)** Challenge Phase – Upon secondary encounter with peanut allergens, the allergens crosslink cell-bound IgE, activating mast cells to release inflammatory mediators. Additional production of IL-4 and IL-13 by mast cells and basophils results in further Th2-cell differentiation and IgE synthesis. Th2 cells and mast cells also produce TNF, IL-5 and chemokines which drive the recruitment of eosinophils to the site of inflammation. This cascade of events drive the release of vast amounts of inflammatory mediators ultimately leading to the induction of symptoms commonly associated with peanut allergy.

Exposure of the immune system to a food allergen usually occurs in the mucosal gut surface. Allergens are taken up by specialized cells, called M cells, and transferred to antigen-presenting cells such as DCs where they are processed into peptide fragments for presentation on the cell surface through a MHC class II molecule. These peptides are presented to naïve T cells resulting, in susceptible individuals, in Th2 cell priming and activation, triggering the cellular and humoral events associated to allergic inflammation. The activation of Th2 cells results in secretion of cytokines such as IL-4, IL-5, IL-9 and IL-13, which stimulate B cells to produce IgE antibodies specific to the allergen. IgE antibodies bind to high affinity IgE receptors, normally expressed on the surface of mast cells and basophils.

In sensitized individuals, subsequent exposure to peanut allergens will trigger an inflammatory response mediated by mast cells, basophils and eosinophils. Crosslinking of IgE on the surface of mast cells and basophils following binding to the allergen leads to the release of several inflammatory mediators including histamine, prostanglandins, leukotrienes, heparin and platelet-activating factor. Further cytokine release occurs at this phase, with further production of IL-4 and IL-13, leading to further polarization towards a Th2 response and IgE synthesis. IL-5 and chemokines such as eotaxin promote eosinophil recruitment to the site of inflammation. Eosinophils are responsible for the release of inflammatory mediators associated with different clinical manifestations like vomiting, diarrhea, and to different allergic diseases such as asthma and anaphylaxis. Even though, mast cells were first thought to be the main effector cells in IgE-mediated acute reaction, further studies have shown that basophils also play a major role in acute food allergy [457].

4.1.1. T cells role in allergic reactions

The critical step for the development of IgE-mediated immune responses is the differentiation of $CD4^+$ T cells, into Th2 effector cells. Th2 responses promote humoral defense to extracellular parasites, recruiting eosinophils and mast cells into the protective immune response[458]. Atopic individuals favor the triggering of Th2 responses, characterized by the release of cytokines like IL-4, IL-5 and IL-13, and IgE production, in response to foreign proteins. Patients with food allergy display a characteristic Th2 phenotype compared to non-atopic controls after *in vitro* stimulation of PBMCs with mitogens or food antigen[459].

Peanut antigen stimulates Th2 cells in peanut allergic donors, while stimulating preferentially Th1 cells in children who have either outgrown their peanut allergy or who are tolerant to peanut, in a response similar to what is observed following stimulation with non-allergenic food antigens[460]. Peanuts contain relatively large quantities of at least 8 proteins that express strong B and T cell epitopes, able to elicit IgE responses[226,227]. Sera from food allergic patients show significant variability with respect to epitope binding. Furthermore, the differential peanut epitope binding of IgE distinguishes those patients with symptomatic peanut allergy from those who either outgrew peanut allergy or who were merely sensitized but clinically tolerant to peanut[461]. Moreover, patterns of epitope binding may correlate with severity of clinical reactions to peanut[462].

During the last decade, Treg cells have received attention because of their ability to suppress effector responses in T-cell mediated diseases, and allergy is no exception. Treg populations may play a role in the development of IgE-mediated allergy by failing to induce or maintain oral tolerance[463,464]. Current data suggest Th2 responses to allergens are normally suppressed by both $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs and Tr1 cells (characterized by IL-10 production), while suppression by these subsets is normally decreased in allergic individuals[465]. Some studies reported an increased frequency of allergen-specific IL-4-secreting cells, and a reduction in the frequency of allergen-specific IL-10 secreting regulatory cells, compared with non-allergic individuals[465]. IL-10 can reduce pro-inflammatory cytokine release from mast cells[466], downregulate eosinophil function and activity, and suppress IL-5 production by human T cells[467]. In agreement, comparing a large cohort of allergic and non-allergic children, cytokine profiles characterized by increased IL-4, IL-5 and IL-13 were associated with allergy, whereas higher IL-10 levels were associated to negative allergy skin tests[468]. In addition, human Th2 clones were relatively resistant to suppression of allergen induced activation by autologous $CD4^+$

$CD25^+$ thymocytes[469]. However, human peripheral blood $CD4^+$ $CD25^+$ T cells suppressed proliferation and Th2 type cytokine production by effector T cells stimulated *in vitro* with allergen[470]. A recent study showed that suppressive ability of Tregs from atopic subjects in co-cultures with allergen-stimulated autologous effector cells, was significantly reduced when compared with cells from non-atopic individuals[471]. These findings suggest that human Treg cells can be functionally defective in atopic subjects. In studies of cow's milk allergy, children who had outgrown cow's milk sensitivity had higher frequencies of circulating $CD4^+$ $CD25^+$ T cells and decreased *in vitro* proliferative responses to bovine β-lactoglobin in peripheral blood mononuclear cells compared with children who maintained a clinically active allergy[216].

Moreover, several murine experiments support an important role for $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells, as well as for IL-10-producing Tr1 cells. For instance, Tr1 clones derived by antigen stimulation, in the presence of IL-10, could prevent Th2 sensitization and IgE production when adoptively transferred prior to sensitization[472]. Tolerance to inhaled antigens can also be induced in mice by high-dose intranasal antigen delivery to the respiratory tract. Such tolerance induction was shown to be dependent on IL-10 production by mature DCs, which induced IL-10-producing Tregs[473]. IL-10 producing Tregs[473] and TGF-β-producing Tregs[474] were also shown to have potent inhibitory activity in airway hyperresponsiveness (AHR) in murine models of asthma. In transgenic mice with monoclonal populations of T and B cells, a single immunization of antigen resulted in very high IgE levels which were prevented by Treg cells[475]. Allergen-specific regulatory T cells modulate the thresholds for mast cells and basophil activation and decrease IgE mediated histamine release[476,477].

Murine models of allergic disease demonstrate that Tregs have the potential to prevent allergic sensitization, however, which regulatory pathways act in overt allergic disease and how these cells suppress fully polarized Th2 responses remains controversial. Treg cells may contribute to the control of allergen-specific immune responses in several ways: (1) through the inhibition of antigen-presenting cells that support the generation of Th1 and Th2 cells; (2) through suppression of Th1 and Th2 cells; (3) by modulating the function on B cells by shifting the predominant allergen-specific isotype from IgE to IgG4 or IgA; (4) by suppressing mast cells, basophils and eosinophils; or (5) by interacting with resident tissue cells thus influencing the tissue remodeling (reviewed in[478]). Active regulation emerges as a key mechanism to maintain and induce peripheral tolerance towards allergens. The ratio between specific Treg cells and Th2 cells can determine the outcome of a healthy or allergic immune response.

4.1.2. Animal Models

Animal models provide a powerful tool to elucidate mechanisms underlying the development of allergy, as well as to establish proof of principle concerning new therapeutic strategies. In the last decade thousands of papers have been published with several different animal models for allergic disease. Although no mouse model fully mimics the full range of clinical manifestations of human allergic diseases, many do reproduce a collection of features that characterize the most common forms, providing a basic core of phenotypic consequences.

Induction of food-induced hypersensitivity in mice is strain dependent. Studies comparing C3H/HeJ with Balb/c mice have shown that C3H/HeJ mice are a highly susceptible strain for food-induced hypersensitivity, developing antigen specific IgE antibodies, and anaphylactic symptoms, when sensitized not only to peanut but also to cow milk, while Balb/c were resistant[218,479]. C3H/HeJ mice represent a good model to study anaphylaxis. These mice develop several clinical manifestations which facilitate the evaluation of disease severity, and ultimately can manifest anaphylactic shock. Moreover, the profound decrease in breathing rate, as well as body temperature drop, are good quantitative measures of disease severity[480]. The development of peptide allergenicity is IgE-mediated, leading to increased IgE systemic levels. Therefore, the main interest of this model, is that it presents objective and quantifiable parameters to monitor the appearance of peanut anaphylaxis. Therefore, C3H/HeJ mice have been widely used to study allergy, namely peanut-induced anaphylaxis[481]. Of note, C3H/HeJ mice are more susceptible to allergy than C3H/HeN, suggesting that the absence of TLR-4 may increase susceptibility to the disease. This may correlate with the finding that TLR-4 agonists favor Treg development[21]. Importantly this model shows several similarities to human anaphylaxis, such as the production of IgE against the main allergenic peanut epitopes (Ara h1 and Ara h2) which are also found in human subjects[453]. Some studies found a correlation between anaphylaxis and increased IgE levels in the serum[480,481]. Moreover, peanut allergenicity was increased compared with cow's milk proteins. Some studies were able to induce tolerance to peanut in C3H/HeJ mice, which was achieved as a consequence of a switch towards Th1 response and increased IFN-γ production[482-484]. C3H/HeJ mice have been also useful for the testing of immunotherapy strategies based on the administration of soy and Chinese herbs for peanut induced anaphylaxis, where peanut-specific response was downregulated[485,486].

In some animal models sensitized to peanut through the oral route it was shown that oral sensitization was dose-dependent, where low dose exposure induced sensitization,

and high dose induced tolerance[487]. In addition, studies in murine models allowed to investigate the consequences of oral immunotherapy, by feeding different quantities of protein for specific tolerance induction, before or after sensitization. These studies have shown that oral tolerance depends on the immune status of the animal, and is controlled by antigen dose, time and frequency of feeding[488-490]. High dose tolerance induction appears to be mediated by lymphocyte anergy or clonal deletion, while low-dose tolerance is suggested to be mediated by Treg cells and anti-inflammatory cytokines such as IL-10 and TGFβ[464]. However, some studies are difficult to compare as they often use distinct sensitization protocols and different strains, requiring optimization in order to lead to increased IgE levels and anaphylaxis[491].

4.1.3. Therapeutic Strategies

The life-threatening nature of anaphylaxis, makes prevention the cornerstone of therapy. Food is the single most common cause of anaphylaxis seen in hospital emergency departments[492], and peanuts or tree nuts cause more than 80% of these reactions[493]. There is no effective method to cure food allergy. Therefore, the management of children with food allergy focuses on the elimination of the causal food from the diet. Future directions in food allergy aim to decrease clinical reactivity after food-allergy is established. Allergen immunotherapy involves the subcutaneous, sublingual or intranasal application of increasing doses of specific allergen extract and is highly effective in some patients with IgE-mediated diseases such as venom anaphylaxis[494]. New routes of allergen administration are now under research, in particular oral administration that was shown effective in desensitizing allergic patients. Although, there are rare successful reports of specific immunotherapy on peanut and fish allergy[495,496], studies in the 1990s indicated that subcutaneous injection immunotherapy was not recommended due to high rate of adverse systemic reactions[497]. In particular in the case of allergy to peanuts and other tree nuts, given the high risk of systemic anaphylaxis allergen immunotherapy is generally avoided.

Data from early-phase clinical trials suggest both sublingual and oral immunotherapy are a promising approach, especially in patients with severe and persistent food allergy, being effective in reducing sensitivity to allergens, even though side effects were frequent but controllable[498]. Successful allergen immunotherapy is associated to a decrease in allergen-specific Th2 response, and the induction of allergen-induced IL-10-secreting Treg cells[499]. Besides, it is generally associated with reduced local nasal infiltration by eosinophils, basophils and T cells after allergen challenge, together with

local increases of T cells producing IL-2 and IFN-γ, detected by in situ hybridization[500]. Some studies have described decreased peripheral blood T cell responsiveness to allergen and/or immune deviation towards Th1 response[501].

Despite the impressive efficacy of allergen immunotherapy with whole allergen extracts it is sometimes associated with significant adverse effects, including anaphylaxis and death. This led to the search for more efficient and safe approaches: recombinant allergen proteins (using the epitope which is known to induce Tregs)[502]; peptide-based allergen preparations, which do not bind IgE and consequently do not activate mast cells, but increase IL-10 levels[503]; moreover the administration of an anti-IgE mAb (omalizumab) together with the allergen immunotherapy, proved beneficial for some patients with peanut allergy by increasing the threshold dose of peanut required to elicit symptoms[504].

Importantly, the increased understanding of the mechanisms involved in tolerance induction, namely the involvement of Treg cell subsets ($Foxp3^+$ and Tr1), led to a shift in the focus of treatment and prevention towards tolerance induction[505]. Even though oral immunotherapy is based on tolerance induction, several other approaches could be exploited, namely the co-receptor or co-stimulatory blockade, which have already been tested in other allergic diseases, and are known to induce immune tolerance in several immune-mediated diseases[506,507]. Moreover, if allergic disease is due to a lack of allergen-specific Treg cells, then effective therapies for allergy should rely on the development of effective Treg cells. Several mAbs targeting co-receptor and co-stimulatory molecules, have been tested in allergic diseases, but were never exploited in the context of food allergy or anaphylaxis. Studies in our laboratory have established that non-depleting anti-CD4 mAb induces antigen-specific immune tolerance in a murine model of airways hypereactivity (Água-Doce, unpublished data). We investigated whether non-depleting anti-CD4 mAbs were effective in tolerance induction in a murine model of severe systemic allergic disease, such as anaphylaxis.

4.2. Material and Methods

Mice. C3H/HeJ mice were bred and maintained under SPF conditions at the Instituto Gulbenkian de Ciência. Experimental animals were between 6-10 weeks of age and sex matched. All experiments involving animals were approved by the Animal User and Ethical Committees at the Instituto Gulbenkian de Ciência, according with directives from Direcção Geral Veterinária (PORT 1005/92). Mice were bred and maintained under SPF conditions.

Sensitization, treatment and challenge. C3H/HeJ mice were given 0.5 mg crude peanut extract (CPE) in 2 mg alum i.p, at days 1, 7 and 21. 1 mg anti-CD4 monoclonal antibody (clone YTS177) was injected i.p on the day before and after each sensitization. Mice were challenged on the indicated day, with 10 mg CPE diluted in PBS i.p. Some experimental groups were given anti-CD25 mAb (PC61 clone) after anti-CD4 period of treatment, and before resensitization.

Clinical assessment of Anaphylaxis. Mice were assessed during 45 minutes following challenge with CPE. Body temperature was measured at regular intervals (rectal thermal probe). The clinical score was evaluated as described elsewhere[481]: 0 – no symptoms; 1 – Scratching/rubbing around the nose and head; 2 – puffiness around eyes and mouth; reduced activity; diarrhea; pilar erecti; 3 – wheezing, labored respiration; cyanosis around mouth tail; 4 – no activity after prodding, tremor, convulsion; 5 – death.

CPE preparation. Peanut was grounded using a stainless steel coffee grinder (Taurus). Following extensive defatting with diethyl ether, the dried peanut flour was ressuspended in ice-cold PBS and mixed overnight at 4º C. The extract was clarified by centrifugation and frozen in aliquots.

Antibodies. Non-depleting ani-CD4 (YTS177), the isotype control (YKIX302), anti-CD25 (PC61) mAbs were produced in our laboratory using integra flasks (IBS, Chur, Switzerland), purified by 50 % ammonium sulfate precipitation, dialyzed against PBS, and purity checked by native and SDS gel electrophoresis. The hybridomas were generously provided by Professor Herman Waldmann (Oxford, UK).

Cell Culture and supernatants. Spleen cells were harvested and red blood cells lysed. Splenocytes ($1x10^6$) were cultured for 3 days in 96 well plates, with complete culture medium (RPMI-1640 with Glutamax, supplemented with 10 % FBS, 1% hepes, 1% penicillin/streptomycin, 1% sodium pyruvate, 0.1% β-mercaptoethanol (invitrogen) and 20 µg peptide (OVA or CPE)). At day 3, cells were centrifuged and supernatants recovered and kept at -80º C until cytokine quantification.

ELISA. Total IgE and CPE or OVA-specific IgG1 were measured in the sera. Microtitre plates were coated with 50 µg/ml CPE or OVA. Capture and detection of IgE antibodies were performed with Opteia kits (BD Pharmingen) and IgG1 with a kit from Southern Biotech. The quantification of cytokines in the cell-culture supernatant was performed using IL-10, IL-13 kits (Prepotech, London, UK) and IL-5 Opteia kits (BD Pharmingen). All assays were performed according to the manufacturer's instructions.

Flow Cytometry. Single cell suspensions were washed in PBS with 0.01% NaN3, 2% FBS. Flow cytometric staining was performed with the following fluorochrome-labeled monoclonal antibodies: CD3 Percp-Cy5.5 (145-2C11), CD4 PE (GK1.5), CD8 APC-Cy7 (53-6.7), CD25 Pe-Cy7 (PC61.5). After staining of surface markers, cells were fixed and permeabilized using Fixation and permeabilization kit from ebiosciences, according to manufacturer's instructions. Foxp3 (FJK165) antibodies were purchased from ebiosciences.

Statistical Analysys. Statistical comparisons were calculated using the two-tailed non-parametric Student's t test (Mann-Whitney U). *P* values < 0.05 were considered to be statistically significant.

4.3. Results

4.3.1. CPE induces an anaphylactic response in sensitized in C3H/HeJ mice

C3H/HeJ mice have been widely used as an experimental murine model of allergic diseases, being prone for the development of Th2-like immune responses, namely, peanut-induced anaphylaxis[218,481]. We found that upon i.p. sensitization with CPE-alum, an anaphylactic response was observed following challenge with CPE administered i.p. (Figure 1A). Female mice appeared to display greater disease severity, however, both males and females developed clinical manifestations of anaphylaxis, reaching a state where they do not respond to external stimuli (score 4)(Figure 1B). In addition these mice displayed a sharp drop in body temperature, during the initial 45' following challenge with CPE (Figure 1C). Serum concentration of Th2-driven immunoglobulins, such as IgE and CPE-specific IgG1 were markedly increased in CPE sensitized mice, compared to non-exposed controls (Figure 1D and E, ***p < 0.01*). All of these features make the C3H/HeJ mice, a good model for the study of anaphylactic shock.

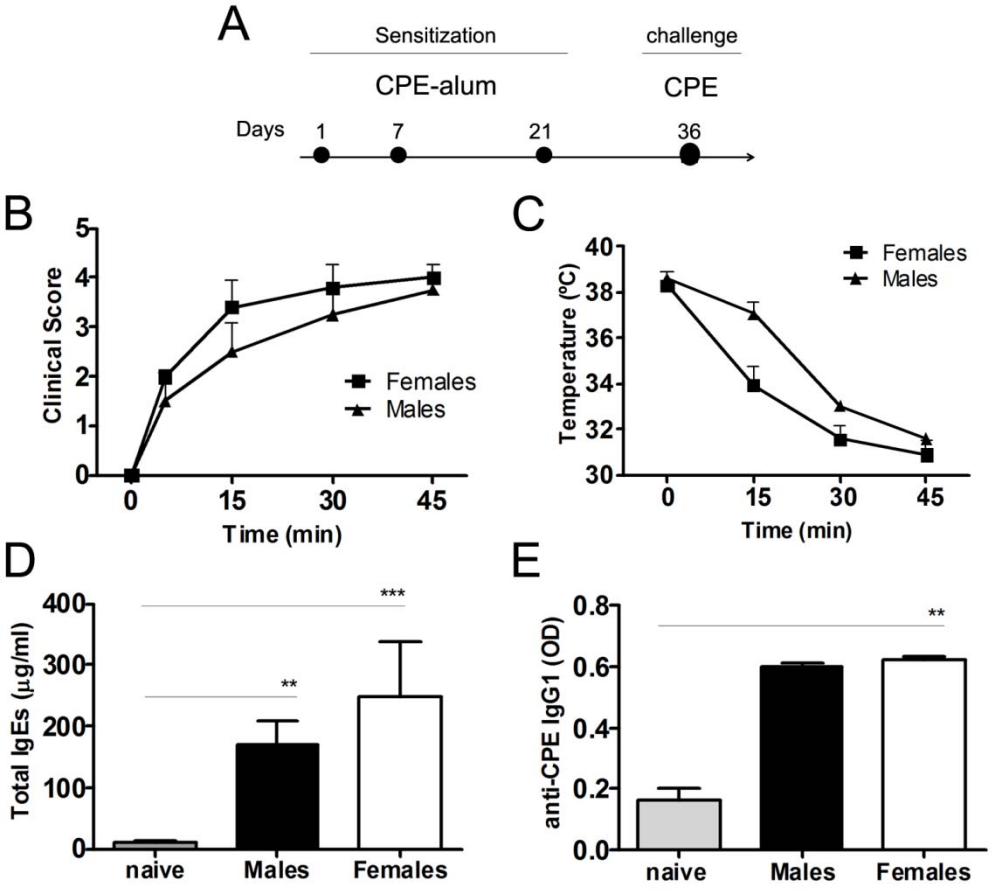

Figure 1 – CPE induces anaphylaxis in C3H/HeJ mice. - **(A)** C3H/HeJ mice were sensitized with 0.5 mg CPE in 2mg alum on days 1, 7 and 21, and challenged with 10 mg CPE i.p on day 36, being assessed for the following 45 minutes. **(B)** Clinical score was evaluated as described elswhere[481], and **(C)** body temperature was measured**.** Females show a more severe allergic reaction, when evaluating both these parameters, compared to male mice. **(D)** Th2-driven immunoglobulins, IgE and (**E)** CPE-specific IgG1 were measured in the sera. Both males and females showed an increase in the immunoglobulins levels compared to naïve mice (***p<0.01*).

4.3.2. Non-depleting anti-CD4 mAb prevents CPE-induced anaphylaxis

Previous studies in our lab, have shown that non-depleting anti-CD4 mAb is effective in preventing allergic airways disease in mice (Água-Doce, unpublished data). To assess if this monoclonal antibody was equally efficient in the prevention of a systemic Th2-driven allergic response, we investigated whether anti-CD4 treatment could prevent anaphylaxis in C3H/HeJ mice. We found that, when giving anti-CD4 at the time of sensitization together with CPE, the treatment was effective, preventing the development of anaphylactic manifestations of diseases, observed during the 45 minutes upon challenge (Figure 2A,B). Moreover, treated mice kept their body temperature stable, compared to untreated mice (Figure 2C). Serum concentration of Th2-driven immunoglobulins was measured. We found anti-CD4 treated mice maintained significantly lower levels of CPE-specific IgGs and IgE than untreated animals (Figure 2 D and E, ****p<0.001*).

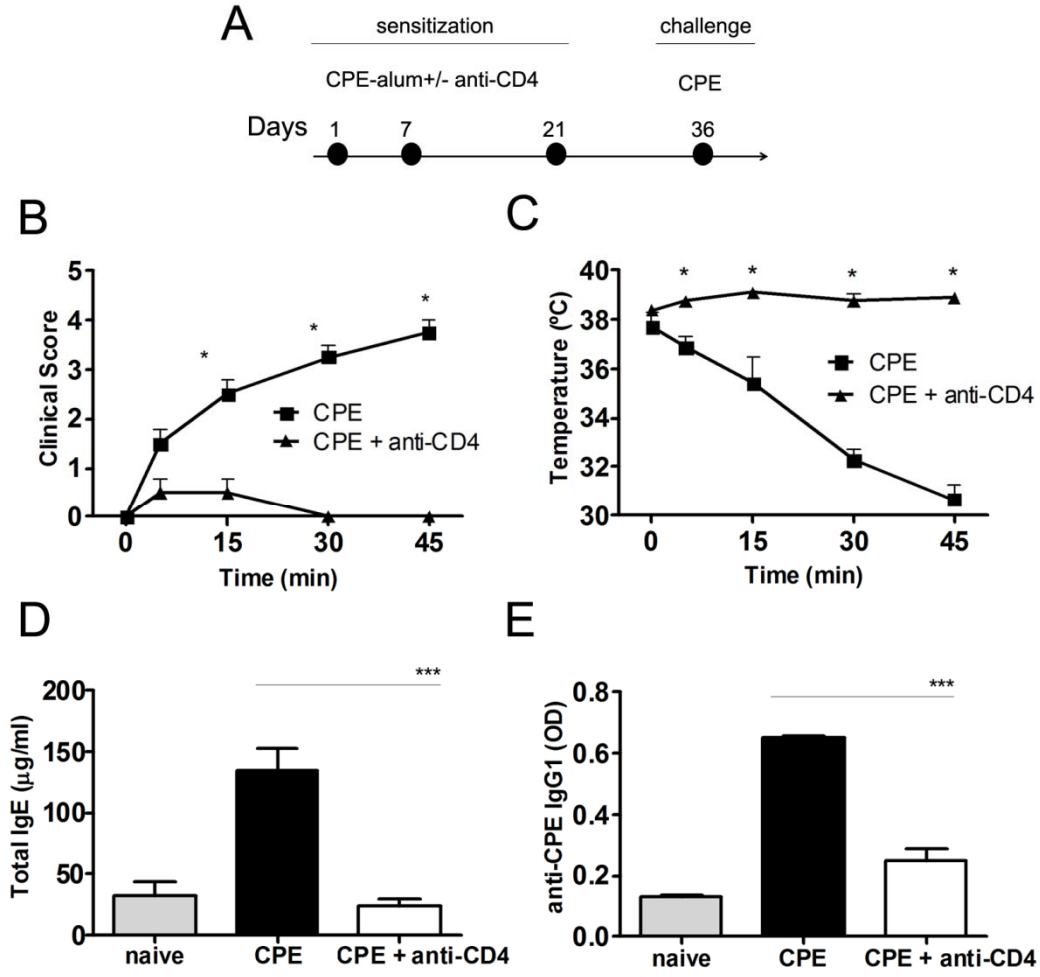

Figure 2 – Anti-CD4 prevents peanut induced anaphylaxis in C3H/HeJ mice - **(A)** Female C3H/Hej mice were sensitized to CPE as described, with a group of animals being treated with 1mg anti-CD4 before and after each sensitization. All mice were subjected to a systemic challenge with 10 mg CPE i.p. **(B)** Mice were assessed for their clinical score and **(C)** body temperature during 45 minutes. Anti-CD4 treated mice did not develop any significant manifestations of disease, compared to the control group *(*p<0.05).* CPE sensitized mice showed a sharp drop of body temperature, while anti-CD4 treated mice kept their body temperature stable *(*p<0.05*). **(D)** Serum concentration of the Th2-driven immunoglobulins IgE and **(E)** CPE-specific IgG1 were measured. Anti-CD4 treated mice maintained their IgE levels at a concentration similar to the naive mice, while CPE sensitized mice significantly increased their serum IgE levels *(***p<0.001*). Serum anti-CPE IgG1 titers from treated mice, were down to the levels of naïve mice, and significantly lower than allergic ones *(***p<0.001*).

4.3.3. Non-depleting anti-CD4 monoclonal antibody induces long term tolerance

Non-depleting anti-CD4 mAb was shown to induce long term tolerance in transplantation[257]. This is a concept of major importance in allergy, since atopic individuals are never free of being re-exposed to the allergen. Therefore, 2 weeks after the first sensitization protocol, mice were re-sensitized to CPE-alum as shown on figure 3A. Systemic anaphylactic manifestations were evident after challenge in CPE sensitized mice, whereas anti-CD4 treated group, did not develop manifestations of allergic disease (Figure 3B), appearing tolerant towards new allergen exposure. The measurement of total serum IgE, and CPE-specific IgG1, confirmed the clinical observations, where tolerant mice immunoglobulins levels remained very close to the baseline observed in naïve non-exposed mice (Figure 3 C).

To compare the specific production of Th2-type cytokines by anti-CD4 treated mice, with or without subsequent re-sensitization with CPE, we cultured spleen cells from different groups for 3 days with CPE stimulation, and quantified the cytokines in cultures supernatants. T cells from mice treated with anti-CD4 mAb did not produce detectable levels of IL-5 and IL-13, while supernatants from control CPE-sensitized mice showed higher levels of these cytokines (Figure 3 D). Several reports have implicated IL-10 in protection from allergy[508]. For instance, non-atopic subjects, as well as bee keepers tolerant to bee venom, have been shown to have greater numbers of IL-10-producing Tr1 cells than individuals allergic to bee venom[465]. Therefore we also assessed IL-10 production by CPE stimulated spleens from both anti-CD4 treated mice and control groups. However, IL-10 levels from culture supernatants were not increased in the anti-CD4 treated group (Figure 3D). In fact IL-10 production was higher in the animals sensitized with CPE in the absence of anti-CD4 treatment.

Several studies in transplantation have shown that long term tolerance achieved through CD4 blockade is associated to Treg expansion[275]. To assess a possible role of $Foxp3^+$ Treg cells in CPE tolerant mice, we analyzed the frequency of $CD4^+$ $Foxp3^+$ T cells in mice treated with anti-CD4 and re-sensitized to CPE. We found that although the anti-CD4 mAb has a non-depleting isotype, the absolute number of $CD4^+$ T cells in the spleen of anti-CD4 treated mice were lower than in controls (figure 3E). However, the frequency of $Foxp3^+$ Treg cells within T cell population was significantly increased in anti-CD4 treated mice (Figure 3E).

To further confirm the participation of Treg cells in protection induced following anti-CD4 treatment, we evaluated the treatment efficacy in CD25 depleted mice. In fact, CD25

is expressed by the majority of Foxp3^{+} Treg cells, and it is still widely used for Tregs depletion studies. CD25 depletion was performed at the time of anti-CD4 treatment, and in advance of CPE re-sensitization. We found that the mice depleted of CD25 T cells were not protected from the allergic disease, exhibiting high levels of total IgE, similar to what was observed in mice not treated with anti-CD4 (figure 3F).

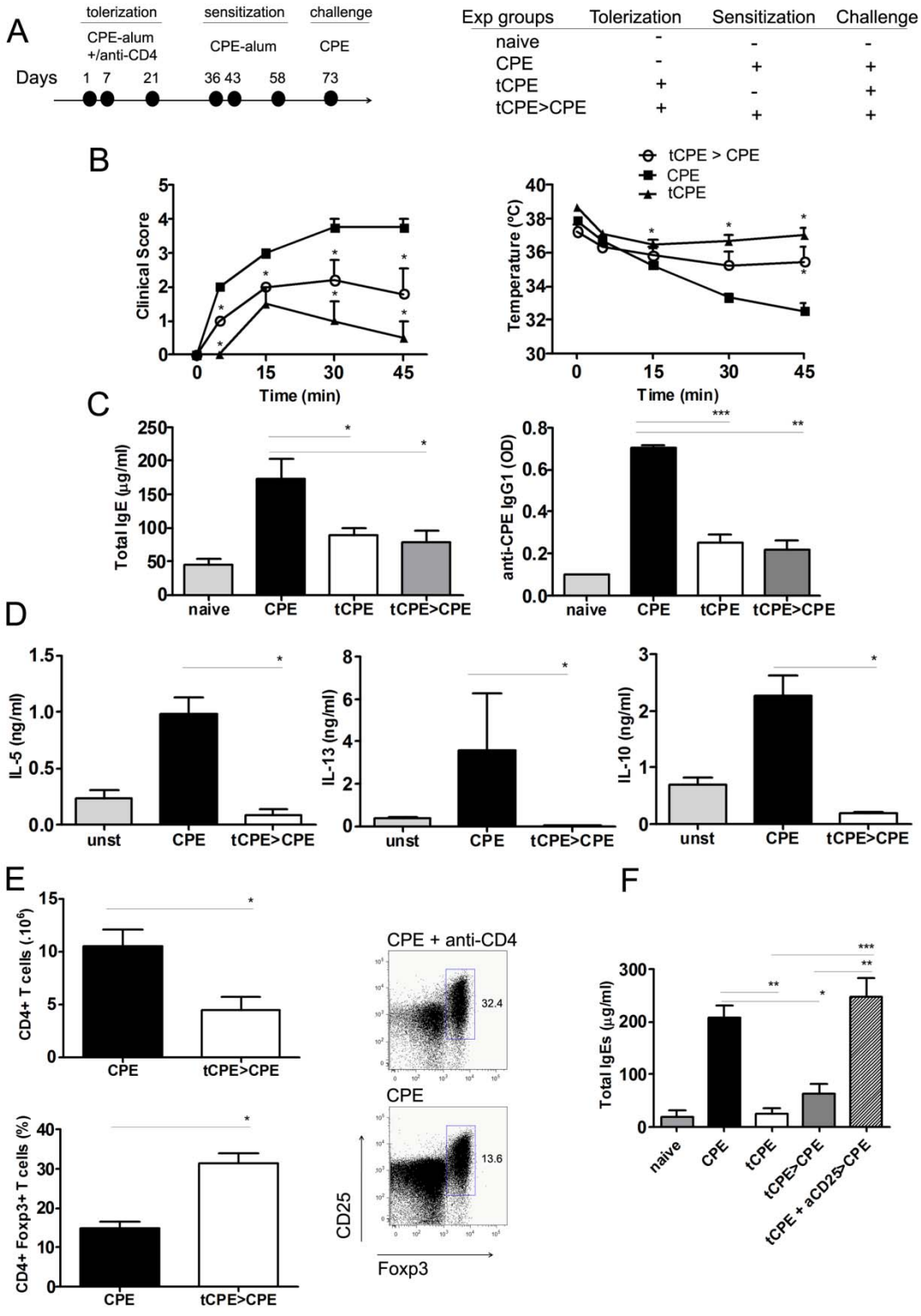

Figure 3 – Anti-CD4 treated mice develop long term tolerance to CPE. - **(A)** C3H/HeJ mice were treated with anti-CD4 (tCPE) at the time of each CPE-alum sensitization, as described in figure 1. Following 2 weeks, the mice were again sensitized with CPE-alum (tCPE>CPE) and finally challenged with 10 mg CPE i.p. **(B)** Mice treated with anti-CD4 (tCPE) even when re-sensitized with CPE-alum (tCPE > CPE) did not reach anaphylactic manifestations as severe as CPE-sensitized group (CPE), showing clinical scores and body temperature significantly different (*$P<0.05$). **(C)** All groups of mice treated with anti-CD4, showed no significant increase in IgE and CPE-specific IgG1 levels when compared to naïve mice. The Th2-driven immunoglobulins were significantly increased in CPE-sensitized mice (*$P<0.05$, ***$P<0.001$). **(D)** Cytokines were measured in the supernatants of CPE-stimulated spleen cells. IL-5, IL-13 and IL-10 were almost undetectable in anti-CD4 treated re-sensitized mice, compared to the CPE group (*$P<0.05$); IL-10 were increased in CPE-sensitized mice, and almost undetectable in mice treated with anti-CD4 prior to re-sensitization (*$P<0.05$). **(E)** The number of splenic CD4+ T cells was reduced in anti-CD4 treated mice, while the frequency of Foxp3+ CD4+ T cells was significantly higher (*$P<0.05$). **(F)** C3H/HeJ mice were treated as described above, while some animals were depleted of CD25 cells at the time of anti-CD4 treatment (tCPE+aCD25>CPE). CD25 depleted mice had significantly higher total IgE levels than mice treated with anti-CD4 in the absence of CD25 depletion (tCPE ***$p<0.001$ and tCPE>CPE **$p<0.01$), reaching levels as high as animals not treated with anti-CD4.

4.3.4. Anti-CD4 tolerance induction is antigen specific

We also assessed whether anti-CD4 treatment was affecting the global immunocompetence, by studying the ability of mAb-treated mice to respond to a different antigen. Therefore, after CPE sensitization in presence of anti-CD4, some mice were re-sensitized, with the same (CPE) or a different antigen (ovalbumin, OVA). Anti-CD4 treated mice, remained fully competent to respond to sensitization with OVA, developing a Th2 immune response leading to high IgE production (Figure 4A). In fact, the levels of IgE were comparable to what was observed in CPE-sensitized control mice (Figure 4 A).

Conversely, when we treated mice with anti-CD4 at the time of sensitization with OVA, we prevented production of IgE following subsequent sensitization with OVA (Figure 4 B).

Taken together these data show that anti-CD4 treatment induces tolerance to the antigens administered at the time of CD4-blockade, without preventing subsequent immune responses to different antigens.

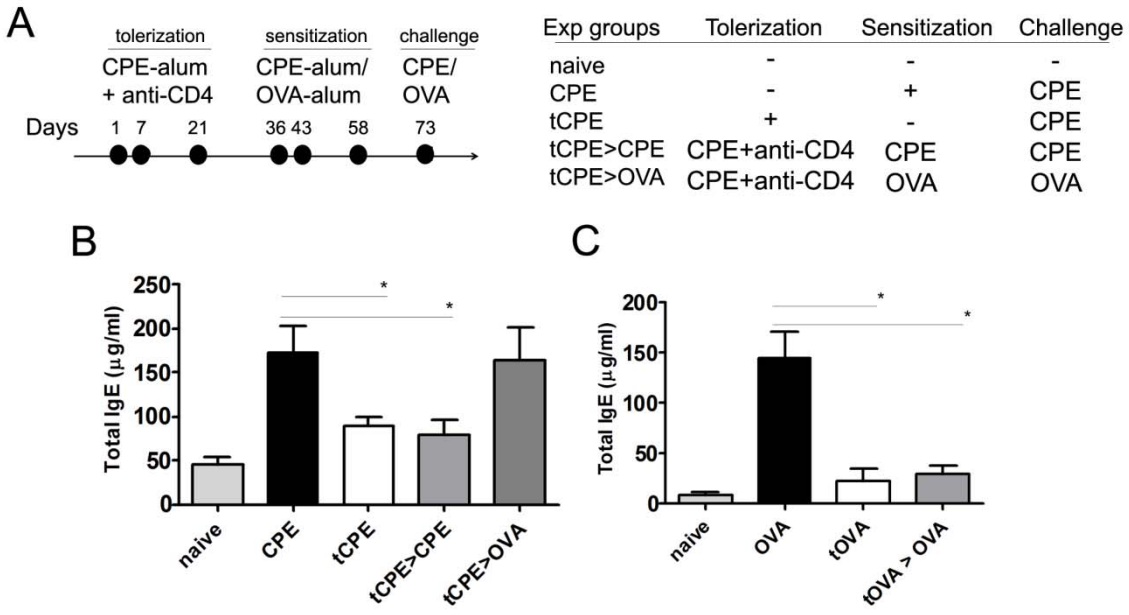

Figure 4 - Anti-CD4 induces antigen-specific tolerance. - (A) C3H/HeJ mice were sensitized to CPE and treated with anti-CD4 as previously described. **(B)** Mice treated with anti-CD4 mAb (tCPE and tCPE>CPE) maintained IgE titers significantly lower than control animals (CPE) (*$P<0.05$); Sensitization of anti-CD4 treated mice with a different antigen – OVA (tCPE>OVA), led to IgE titers similar to the levels of CPE-sensitized untreated mice (CPE). **(C)** C3H/HeJ mice were sensitized to OVA, and treated with 1 mg anti-CD4 before and after each sensitization, as described before (tOVA); some mice were subsequently sensitized with the same antigen (tOVA>OVA). All mice treated with anti-CD4 regardless of additional OVA-sensitization maintained low IgE levels significantly lower than OVA-sensitized control group (OVA) (*$P<0.05$).

4.3.5. Anti-CD4 is not able to prevent the disease in pre-sensitized mice

Finally we assessed the impact of anti-CD4 treatment in pre-sensitized mice. Note that desensitization, probably due to alum induces a mild clinical manifestation of disease (day 36, not shown), making it possible to investigate desensitization. We found that mice sensitized with CPE in advance of treatment were not protected from the development of clinical manifestations of anaphylaxis, namely reduction of body temperature (Figure 5B and C). These mice had increased levels of total IgE and CPE-specififc IgG1 similar to CPE-sensitized controls (figure 5D and E). These results confirm that in pre-sensitized individuals pre-existing IgE will drive clinical manifestations of anaphylaxis in a T-cell independent manner. It remains to be shown whether therapeutic neutralization of IgE in sensitized individuals allows tolerance to be imposed in a way preventing further production of IgE.

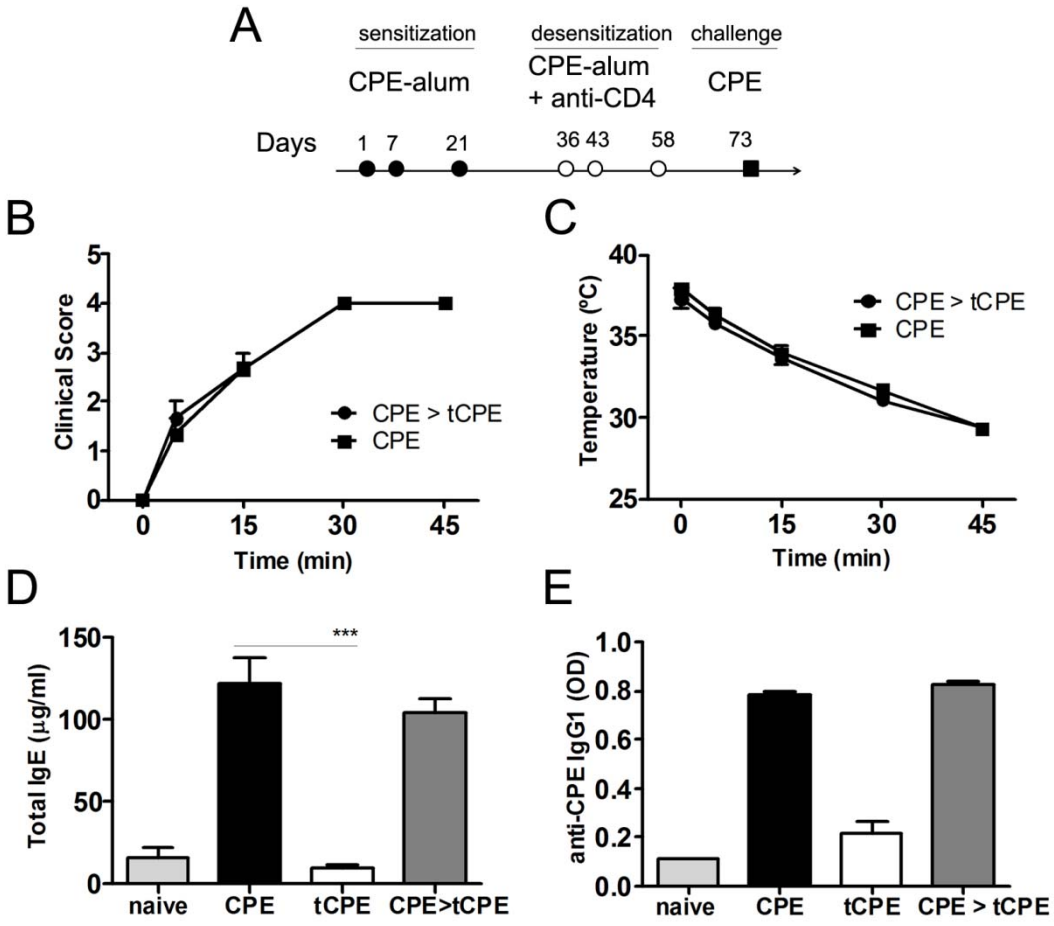

Figure 5 – Anti-CD4 does not prevent anaphylaxis in pre-sensitized mice. - **(A)** C3H/HeJ mice were sensitized to 0.5 mg CPE-alum on days 1, 7 and 21, and treated during a second sensitization protocol, 1 mg anti-CD4 before and after each sensitization shot, being then challenged with 10 mg CPE i.p. **(B)** Anti-CD4 treated mice after sensitization (CPE>tCPE) displayed severe anaphylatic manifestations, as well as **(C)** sharp body temperature drop, similar to CPE-sensitized mice **(D)** Total IgE and **(E)** CPE-specific IgG1 levels were measured in the serum of CPE-sensitized group, anti-CD4 treated (tCPE) and pre-sensitized anti-CD4 treated mice (CPE>tCPE). Both Th2-driven cytokines were up to the levels of the CPE-sensitized group.

4.4. Discussion

Non-depleting anti-CD4 mAb has been extensively studied in several experimental models for tolerance induction, namely in transplantation, where tolerance has been shown to be dependent on Treg cells expansion[275,350]. Studies in our lab have shown that CD4 blockade was able to fully abbrogate the Th2 response in a mouse model of allergic airways hyperreactivity (Água-Doce, unpublished data). We now investigated if this same antibody treatment would be equally able to prevent a Th2 response pushed to the limit – an anaphylactic response.

The C3H/HeJ mice have been widely used due to their susceptibility to allergic responses. Furthermore, anaphylactic reactions in these mice model mimic some clinical characteristics of human anaphylaxis[481]. We found CD4 blockade treatment was able to prevent peanut-induced anaphylaxis in C3H/HeJ mice. The treatment efficacy was evident in reducing the clinical manifestations of the disease, and also preventing the decrease in body temperature. In addition, Th2-driven immunoglobulins, such as IgE and CPE-specific IgG1, remained down to the levels of naïve mice in anti-CD4 treated mice. Finally, T cells from anti-CD4 treated mice produced less or none Th2 type cytokines such as IL-5 and IL-13.

Moreover, treatment with anti-CD4 mAb, besides preventing disease development, when given at the time of sensitization, was able to induce long-term tolerance to CPE. When subjecting mice previously treated with anti-CD4, to a second sensitization protocol, the animals remained protected from anaphylaxis. Moreover, mice protected from CPE-induced anaphylaxis remained fully competent to mount a normal immune response when sensitized to a different antigen. Importantly, we did not detect Th1 type cytokines in supernatants of stimulated spleens from anti-CD4 treated mice (data not shown). Therefore tolerance induction is unlikely to be due to a Th1immune deviation, as it has been described in several protocol of specific-immunotherapy[500,501,509]. Furthermore, responses to OVA were prevented, if OVA was used at the time of anti-CD4 treatment, instead of CPE, showing that the tolerance induction is antigen-specific.

Both natural and induced Treg cells are known to play important roles inhibiting allergen-specific effector T cells in experimental models[508,510]. In addition, the development of allergy might be due to insufficient development of allergen-specific Treg cells expressing Foxp3 in atopic individuals[511]. Given the importance of Treg cells in the maintenance of oral tolerance, as well as mediating tolerance induction in murine models of allergy, we evaluated Treg role in our model. We found, anti-CD4 treated mice had increased levels of $Foxp3^+$ Treg cells within the $CD4^+$ T cell population, suggesting a role

for Treg cells in the development of long-term tolerance to CPE. This is in agreement with previous reports in transplantation, where long term tolerance achieved upon anti-CD4 treatment is dependent on Treg cells [163]. Supporting this observation, depletion of $CD25^+$ cells in low dose peanut oral tolerance prevented tolerance induction, suggesting an important role for Tregs in regulating hypersensitivity responses[512].

To address Treg cells involvement in anti-CD4 treated mice, we depleted CD25 cells at the time of anti-CD4 treatment. We found that $CD25^+$ cells depletion was sufficient to abrogate tolerance induced with anti-CD4, supporting an important role for Tregs as mediators of long-term tolerance. Of note, although IL-10-producing Tr1 cells have been identified to maintain tolerance in several allergy studies, we did not find evidence for increased IL-10 production by T cells from anti-CD4 treated mice.

It is well established that sensitized animals have high allergen specific IgE titers in circulation, as well as memory B cells able to produce IgE when re-exposed to the allergen. Besides, some IgE molecules are linked to FcRI high affinity receptors on mast cells, leading to their degranulation upon allergen crosslinking. Therefore, it is not surprising that anti-CD4 treatment could not prevent anaphylaxis in pre-sensitized mice. Interestingly, it appears that CPE-alum is less prone to induce anaphylaxis in sensitized mice than CPE without adjuvant.

An efficient and specific therapy for anaphylaxis, namely to peanuts and other tree nuts has been a major unmet medical need. Once the diagnosis of allergy is established, the only effective measure to avoid anaphylaxis is the strict elimination of the exposure to the allergen, which is not always possible. For instance, it was estimated that more than 50% of individuals who are allergic to peanuts will have an accidental exposure over a 2-year period[451]. Earlier experiments regarding desensitizing patients either orally or with subcutaneous injections of food extracts were not conclusive[513,514]. Even though, novel immunotherapeutic strategies designed to alter the immune system's response are being examined as potential treatment modalities for food allergy, nothing has yet been approved. Taken together, our data show anti-CD4 immunotherapy seems effective in preventing sensitization leading to anaphylaxis. Furthermore, the effects are antigen-specific and spares overall immune competence. The protective effects are long-term, extending beyond the half-life of the therapeutic mAb, possibly due to the action of allergen-specific Treg cells. It remains to be shown whether measures to eliminate pre-formed IgE may allow tolerance induction in a pre-sensitized immune system. Our data from previous chapters, suggests this goal may be attainable.

General Discussion

5. General Discussion

In the last 30 years Mabs arose as potential and, in several cases, efficient therapeutic tools. The induction of peripheral tolerance has been a promising area of research, with successful cases in animal models of transplantation and autoimmunity. Several tolerance-inducing Mabs work by targeting co-receptor and co-stimulatory molecules on the system cell – APC interface (for instance CD3, CD4, CD40L, CTLA-4, OX40L), thus interfering with the immune synapse[515]. T cells have been described as major players in immune-mediated pathologies such as autoimmune diseases and transplant rejection. Therefore, mAbs depleting T cells were among the first initially tested for the treatment of those diseases[242,245]. In transplantation, it soon became apparent that tolerance to foreign proteins could be achieved without T-cell depletion, being CD4 co-receptor blockade sufficient for that purpose[163,247]. More recently, anti-CD4 mediated tolerance was shown to be dependent on Treg induction[275], and since then, regulatory T cell-mediated peripheral tolerance has been achieved in several animal models of transplantation, autoimmunity, and allergy[71,167,252,440,478].

We studied the effect of non-depleting anti-CD4 in immune-mediated pathologies where T cells were shown to have a pathogenic role, such as autoimmunity and allergic diseases. $CD4^+$ T cells can differentiate into specialized functional subsets, which drive different types of immune responses. Th17 and Th1 cells have been associated to autoimmune pathologies, while allergic diseases and anaphylaxis are Th2-driven responses. Therefore, we addressed the impact of CD4-blockade in the development of diseases driven by different T helper subsets and affecting different organs, such as the joints in the case of autoimmune arthritis, the CNS in the case of multiple sclerosis, and in a systemic anaphylactic response.

CD4 blockade was able to prevent disease development in chronic autoimmune arthritis in SKG mice, and impaired its progression to severe disease. Importantly, SKG arthritis models several aspects of human rheumatoid arthritis[194]. We found that the mechanism through which anti-CD4 was abrogating disease development was by affecting Th17/Treg ratio within the synovia[443]. TGF-β is known to be an important cytokine for the differentiation of both Th17 and Treg cells, and it is likely that CD4-blockade in presence of TGF-β could shift the differentiation of activating T cells from Th17 to Treg[55,57]. Moreover, it was shown T cells may interconvert from Th17 towards Tregs and vice-versa, depending on the anti- or pro-inflammatory environment they encounter[81,89].

In order to further examine how CD4-blockade modulates pathogenic effector T cells

we had to use a different experimental system as SKG autoimmune arthritis had the disadvantage that the peptides triggering the immune response are still unknown, thus making it harder to study the antigen-specific T cell response.

At this point we knew that somehow CD4-blockade was affecting the balance between effector and Treg cells, locally, at the site of inflammation. We next investigated the mechanism through which the anti-CD4 MAb was affecting this Teffector/Treg ratio. *In vitro* studies in our laboratory showed that non-depleting anti-CD4 mAb is able to induce Foxp3$^+$ CD4 T cells from naive T cells specific to a certain antigen by interfering with the T cell activation threshold (Oliveira et al, submitted). We took advantage of a model of multiple sclerosis, the MOG-induced EAE, in combination with different TCR-transgenic mouse strains bearing T cells specific to neural antigens, to follow antigen-specific effector T cells as tolerance is induced. Therefore, the study of EAE in C57Bl/6 mice model, in a MBP-specific TCR transgenic mice in a RAG-deficient background, and following adoptive transfer of 2D2 MOG-specific TCR-transgenic cells, allowed a better understanding of the impact of anti-CD4 treatment in the antigen-specific T cell population.

Anti-CD4 treatment prevented the disease development in all studied animal models of EAE and, more importantly, impaired disease progression in mice treated following the early manifestations of EAE. We found that anti-CD4 affects naive T cells by inhibiting their proliferation and differentiation towards pro-inflammatory function at the time of disease induction, and at a later time point, CD25$^+$ cells were shown essential for tolerance maintenance. In fact, in TR- mice, which do not have Foxp3$^+$ T cells, anti-CD4 treatment induces the *de novo* generation of Foxp3+ Treg cells. However, anti-CD4 acts differently on pre-activated T cells by inducing apoptosis, and consequently, preventing CNS infiltration.

Finally, we studied this same treatment, which we have shown effective in both models of autoimmunity, in a murine model of anaphylaxis. We know from studies from our laboratory, treatment with anti-CD4 is able to prevent allergic airways hyperreactivity, and induce antigen-specific long-term tolerance (Água-Doce et al, unpublished data). Anaphylaxis is a severe systemic Th2-triggered response which can lead to death within minutes. C3H/HeJ mice have been used as a model of peanut-induced anaphylaxis, due to their similarities to human disease, namely the production of immunoglobulins targeting the same peanut epitopes[481]. Moreover, the development of severe anaphylaxis upon CPE challenge, with a sharp drop in body temperature, allows an easy and immediate evaluation of disease severity. We found anti-CD4 treatment was able to prevent anaphylactic manifestations in C3H/HeJ mice when the Mab was

administered at the time of sensitization. Tolerance induction following CD4-blockade was long-term and antigen-specific, with treated mice remaining immune competent to respond to different antigens. We found Foxp3+ Treg cells are key players in the maintenance of tolerance. This is in agreement with several studies supporting an important role for Treg cells in allergy protection[478], and with the fact that anti-CD4 treatment favors peripheral Treg induction in transplantation studies[275]. In contrast, data from our laboratory concerning tolerance induction with anti-CD4 Mab in a murine model of allergic airways hiperreactivity, suggests tolerance induction is independent on Foxp3+ T cells (Água-Doce, unpublished data). The reason for those differences has yet to be established.

Overall, anti-CD4 was effective in the prevention of both autoimmune and allergic pathologies, independently on the pathogenic T helper subsets involved. These data support the potential use of non-depleting anti-CD4 mAb in different immune-mediated diseases, proven to be dependent on $CD4^+$ T cells. Although different anti-CD4 MAbs have been tested in clinical trials of autoimmune diseases such as RA, the immaturity of the field at that time may have led to premature dismissal of potentially useful drugs. This was probably the case for the anti-CD4[266-269,328].

The major objective for a given therapy, is the ability to revert established disease, as most autoimmune and allergic diseases are not accurately predictable, being only diagnosed upon initial clinical manifestations. Therefore, an ideal treatment should not only prevent the onset but importantly, also treat established disease. Our results show anti-CD4 treatment can be effective in established disease, as it was able to impair disease progression in autoimmune arthritis and especially in EAE. In fact, anti-CD4-treatment was able to revert the clinical manifestations of EAE in TR- and following the adoptive transfer of pre-activated 2D2 TCR-transgenic cells, conditions that rapidly led to fatal outcome in the control animals. This was an important observation as it is generally assumed mAbs targeting CD4 are mostly effective in preventing the onset of diseases. The specificity of tolerance state induced following CD4-blockade, as well as the maintenance of tolerance for long-term tolerance in the absence of subsequent anti-CD4 administrations stand as important features of this tolerogenic strategy. The exquisite antigen-specificity allowed the maintenance of a competent immune system, able to withstand challenge with a gamma herpes virus. In fact, these observations contrast with the current most common alternatives for the treatment of autoimmune diseases, mainly based on non-specific immunosuppressive and anti-inflammatory drugs (including Mabs), with a short-term effect often leading to relapse once the drug is withdrawn, and the usual complications associated to immunosuppression, including life-threatening

infections and tumors. The alternative is aiming at antigen-specific tolerance, silencing the pathogenic response while keeping host defense mechanisms largely intact. This appears to be the case for anti-CD4 therapy, as mice were able to mount a competent immune response when immunized to different antigen in all animal models tested.

The versatility of mechanisms induced by anti-CD4 treatment seems to depend on several factors. Non-mitogenic anti-CD3 mAbs were also described to induce T cell anergy[516], $CD4^+CD25^+$ Treg cell production of TGF-β[440] and long-term tolerance[124]. Our data also show the same MAb may differ in its tolerance-inducing effectiveness due to different contexts (namely prior-sensitization or local versus systemic distribution of the antigen). Furthermore, the mechanisms leading to tolerance may change according to those diverse inflammatory conditions.

However, it seems the common denominator for long-term tolerance relies on the induction of Treg cells, and the control of pre-existing effector T cells. Further studies need to be performed to better characterize tolerance induction by anti-CD4, as well as all the molecular mediators favoring Treg induction. We hypothesized suboptimal recognition of the antigen may trigger Treg commitment by naive T cells[448] (Oliveira *et al*, submitted). For instance, several studies support a role for Tregs in tolerance induction by low dose antigen exposure, while high dose mostly induces activation induced cell death[517]. Persistent exposure to the antigen also seems to facilitate Treg induction, namely in transplantation[275]. However, it should be noted CNS antigens, although persistent, are not readily accessible by lymphocytes due to the BBB. Moreover, the presence of a pro-inflammatory environment, for instance provided by an adjuvant may also determine different tolerance outcomes (Água-Doce, unpublished data). Importantly, EAE induction often requires administration of an adjuvant (CFA), while allergic sensitization prior to induction of anaphylactic shock is induced with a CPE emulsion in Alum. It is likely that several of the above mentioned factors contribute to the overall environment present at the time T cells are activated. The way CD4-blockade modulates this event is not yet fully understood.

Following anti-CD4 success in several studies in experimental animals[239,259,325,506], this Mab was among some of the first MAbs entering clinical trials [267,268,326,447]. However, the results of those trials were not satisfactory due to lack of efficacy and the presence of several adverse effects. With hindsight we can see those disappointing results were to a great extent a consequence of the early development of the field. In fact, the clinical trials were initiated, given the promising results from animal models, at a time therapeutic MAbs had not yet matured. Those clinical trials were conducted prior to the development of the first humanized MAb, therefore the infused MAbs were non-humanized and highly

immunogenic molecules. In fact, it was documented that the lack of response in RA trials were due to the emergence of immunoglobulins targeting the therapeutic molecules[518,519]. In addition, several studies were conducted with anti-CD4 MAb that caused some degree of T cell depletion. As a consequence it was not possible to achieve efficient CD4-blockade without incurring in significant lymphopenia that was not acceptable[520]. Recently, it was developed a humanized CD4 antibody with low immunogenicity[521]. In a similar way, early trials with anti-CD3 mAb were associated to significant adverse effects[522]. However, the potential for CD3 targeting has been re-evaluated in diabetes following the development of a modified molecule that does not lead to cytokine release syndrome [253].

Moreover, we know there are several outstanding issues concerning the efficacy of therapeutic MAbs that require optimization, and may account for differences between animal models and clinical use. These may be for instance the MAb dose and epitope targeting. Different antibodies may have differential functional effect depending on the particular epitope recognized and the Fc-mediated effector function elicited by MAb. Some MAbs may induce depletion through antibody-dependent cell-mediated cytotoxicity (ADCC) or inducing antigen-dependent apoptosis, others block signals affecting different pathways, or may inhibit T cell activation[523]. It is important to investigate all these parameters while considering translating pre-clinical data into the clinic.

As a final remark, our data suggest that the therapeutic potential of non-depleting anti-CD4 should be revisited. However, a better understanding of molecular events leading to tolerance induction on T cells under the cover of anti-CD4 may facilitate the rational design of the most effective therapeutic strategy.

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7. APPENDIX